REMARKS

Claims 1-26 are pending and under examination.

The rejection of claims 1-26 under 35 U.S.C. § 112, first paragraph, as allegedly lacking enablement is respectfully traversed. Applicants respectfully maintain that the specification provides sufficient description and guidance to enable the claimed methods.

The Office Action refers to several factors alleged to be related to the failure of developing suitable HIV vaccines. Applicants respectfully submit that these issues are not relevant to the claimed methods. Applicants respectfully submit that, based on the teachings in the specification and what was well known to those skilled in the art, one skilled in the art would have been able to make and use the invention as claimed.

First with respect to meaningful indices for assessing the efficacy of the claimed methods, the Department of Health and Human Services (DHHS) has issued Guidelines developed by the Panel on Clinical Practices for Treatment of HIV Infection for use of antiretroviral agents in HIV-1-infected adults and adolescents. These Guidelines are located at http://www.aidsinfo.nih.gov/guidelines/default_db2.asp?id=50. Attached herewith as Exhibit A are the cover page, pages ii-iv showing the table of contents, and pages 2-8 of the Guidelines. The Guidelines indicate on page 4, under "Initial Assessment and Monitoring for Therapeutic Response," that "[T]wo surrogate markers are routinely used to determine indications for treatment and to monitor the efficacy of therapy: $CD4^+$ T-cell count and plasma HIV RNA (or viral load)." The Guidelines additionally indicate that the "$CD4^+$ T-cell count (or CD4 count) serves as the major clinical indicator of immunocompetence in patients with HIV infection" (page 4, left column, under "$CD4^+$ T-cell count"). The Guidelines further describe a "significant association between a decrease in plasma viremia and improved clinical outcome. Thus, viral load testing serves as a surrogate marker for treatment response and may be useful in predicting clinical progression" (page 4, right column, last paragraph). A $CD4^+$ T cell count >350 cells/mm^3 and plasma HIV RNA (viral load) >100,000 copies/ml is considered to be an indication to defer initiating treatment (see table on page 6). The expense of antiretroviral drugs

and the association of drug resistance and toxicity with antiretroviral therapy clearly indicates that methods of treating an HIV-infected individual that could delay the initiation of antiretroviral therapy and/or extend the time during which antiretroviral therapy can be administered would be clearly advantageous, and the claimed methods provide such advantages by increasing CD4+ T helper cell response (claim 2) and/or decreasing viral load or delaying an increase in viral load.

In corroboration of Applicants' position that the claimed methods are enabled, submitted herewith are several references that describe immune stimulation, increased $CD4^+$ and decreased viral load or delayed increase in viral load in response to HIV immunogen. Submitted as Exhibit B is a reference by Moss et al., Vaccine 21:1066-1071 (2003), which is a publication resulting from continued studies of those disclosed in the specification. The study includes additional subjects than those described in the experiments disclosed in the specification. In the immunized group, HIV antigen stimulated lymphocyte proliferative responses, as did native p24 antigen (page 1068, first paragraph of Results). HIV antigen and native p24 antigen-stimulated MIP-1β levels were significantly increased after immunization and prior to drug discontinuation (page 1068, paragraph bridging columns). There was a trend toward an increased number of subjects with a viral load of less than 10,000 copies at the end of the second structured treatment interruption (STI) compared to the end of the first STI in the immunized group (page 1069, first paragraph). These results show that the immunized group had augmented HIV-specific T helper immune responses and β-chemokines after immunization and prior to antiviral drug treatment discontinuation.

Submitted as Exhibit C is a reference by Lichterfeld et al., J. Exp. Med. 200:701-712 (2004). This reference describes the loss of HIV-1-specific $CD8^+$ T cell proliferation after acute HIV-1 infection and restoration by vaccine-induced HIV-1-specific $CD4^+$ T cells. Individuals were immunized with gp120-depleted, inactivated HIV, and an increase in $CD4^+$ T cells secreting interleukin-2 (IL-2) was observed (page 708, right column, second paragraph). After immunization, HIV-1-specific $CD4^+$ T cells in vaccinees but not control individuals developed strong proliferative capacities and strong lymphoproliferative activities in HIV-1-specific $CD8^+$

T cells (page 708, right column, second paragraph). These results indicate that *in vivo* augmentation of virus-specific $CD4^+$ T cell responses can lead to the reconstitution of HIV-1-specific $CD8^+$ T cell lymphoproliferative immune responses *in vivo* (paragraph bridging pages 709-710).

Additionally submitted herewith as Exhibit D is a reference by Fernandez-Cruz et al., Vaccine 22:2966-2973 (2004). This reference describes that administration of HIV immunogen resulted in a delay in time to reach virologic failure, as measured by an increase in viral load above 5000 copies per milliliter for the two drug combination of zidovudine (AZT) and didanosine (DDI); a viral load above 2000 copies per milliliter for the three drug combination of lamivudine (3TC), stavudine (D4T) and Indinavir (IDV); a decrease of $CD4^+$ count below 250 cells/mm^3; or a decrease of 50% below baseline (page 2968, right column, section 2.5; and page 2969, right column, last paragraph). The data suggest a delay of at least one year in virologic rebound in those receiving HIV immunogen compared to placebo and a 34% decrease in the risk of virologic failure (page 2969, right column, last paragraph, to page 2970, left column; and pages 2971-2972, first paragraph of Discussion).

Also submitted herewith as Exhibit E is an abstract (IAS05) to be presented at the Third IAS Conference on HIV Pathogenesis and Treatment in Rio de Janeiro July 24-27, 2005. This abstract describes the results of a Spanish phase II trial called the REMIT study. The REMIT study enrolled 39 patients who had previously participated in the STIR-2102 Phase II double-blind study (Fernandez-Cruz et al., Exhibit C) in which the patients received either REMUNE™ or Incomplete Freud's Adjuvant (IFA), and then subsequently received REMUNE™ in an open-label extension. For the REMIT study, the patients were randomized to receive either REMUNE™ (n = 21) or IFA placebo (N = 18) at the time of discontinuation of highly active anti-retroviral therapy (HAART). Endpoints in the 48-week study measured immunologic or virologic failure of patients during treatment interruption, defined as time to reach HIV RNA viral load failure (defined as HIV RNA >55,000 copies/mL), time to reach $CD4^+$ failure (as defined as <350 cells/mm^3), and/or time to re-initiation of HAART.

Analysis of the data was performed based on comparisons of four groups composed of patients who had received the following treatments: 1) REMUNE™ in the STIR-2102 and REMUNE™ in the REMIT study (n = 9); 2) REMUNE™ in the STIR-2102 and IFA in the REMIT study (n = 10); 3) IFA in the STIR-2102 and REMUNE™ in the REMIT study (n = 12); and 4) IFA in the STIR-2102 and IFA in the REMIT study (n = 8). All patients received REMUNE™ during the open-label extension phase between the STIR-2102 double-blind study and the REMIT study. The total number of REMUNE™ injections received by the participants ranged from 5 to 28.

The results showed that patients who received REMUNE™ in both the STIR-2102 and the REMIT study were less likely to reach a study failure endpoint in 48 weeks of observation in the REMIT study compared to the other three groups. The median number of REMUNE™ doses received in this group was 27. The number of doses of REMUNE™ received was identified as a prognostic factor in predicting delay of virologic failure during treatment interruption, as was baseline $CD4^+$ count prior to initiation of antiretroviral therapy. There was also a positive correlation between magnitude of HIV-specific immune responses and ability to delay virologic failure in these patients.

Further submitted herewith as Exhibit F is another abstract, Gori et al., also to be presented at the Third IAS Conference on HIV Pathogenesis and Treatment in Rio de Janeiro July 24-27, 2005. This abstract describes the results from a phase II study conducted in Italy. The multi-center, single-blind, randomized study followed 51 patients over 28 weeks following treatment with REMUNE™, IFA or saline. Patients were antiretroviral therapy naive and had HIV RNA levels between 10,000 and 40,000 copies/mL and CD4 counts between 400 and 800 cells/μL at study entry. Patients received three injections of REMUNE™ (n=19), IFA (n=10), or saline (n=11) at weeks 0, 12, and 24. A fourth group received only a single injection of REMUNE™ at week 0 (n = 11). The final analysis included data from 51 patients enrolled in the study, and showed that median absolute CD4 cell counts remained stable through week 28 in the patients that received 3 injections of REMUNE™, but declined in both the IFA and saline groups. The effect of REMUNE™ on immune reconstitution, as further evidenced by an

augmented serum concentration of IL-7 and increases in naive $CD4^+$ T cells, suggests a possible mode of action via stimulation of thymus function.

As for the issues raised in the Office Action regarding the factors alleged to contribute to failure of identifying and developing suitable HIV vaccines, Applicants respectfully submit that these issues are not relevant to the claimed methods. As discussed above, the Department of Health and Human Services recognizes that $CD4^+$ T cell levels and viral load are both suitable surrogate markers to assess the effectiveness of therapy and disease progression. The specification teaches that the invention methods stimulate $CD4^+$ and $CD8^+$ anti-HIV immune responses (page 5, lines 3-7). Furthermore, the evidence submitted herewith shows that an HIV immunogen successfully increased $CD4^+$ T cell count (Exhibit F) and delayed increase in viral load (Exhibits D and E) and therefore clearly demonstrates measurement of meaningful immunological and virological indices of efficacy of the claimed methods.

With respect to the alleged lack of suitable immunogens, adjuvants, routes of administration and immunization regimens, Applicants respectfully submit that, based on the teachings in the specification and what was well known to those skilled in the art, one skilled in the art would have been able to use suitable immunogens, adjuvants, routes of administration and immunization regimens for the claimed methods. For example, the specification teaches exemplary HIV immunogens, adjuvants and immunization regimens (for example, on page 12, line 3, to page 18, line 2). That such immunogens, adjuvants, routes of administration and immunization regimens would be well known to those skilled in the art is corroborated by the Exhibits submitted herewith, which describe successful methods of treating an HIV-infected individual with an HIV antigen, including with structured treatment interruption (STI), resulting in increased $CD4^+$ T helper cell responses and/or decreasing viral load or delaying an increase in viral load.

Regarding the assertion that there is a lack of adequate animal models reasonably predictive of clinical efficacy, Applicants respectfully submit that this is not relevant since the specification discloses human clinical trial data, not an animal model. Furthermore, the Exhibits submitted herewith all relate to human clinical trial data. Without addressing the merits of

whether suitable animal models exist, Applicants nevertheless maintain that this issue is irrelevant in light of the teachings in the specification and the corroborative evidence submitted herewith.

Based on the teachings in the specification, and corroborated by the evidence submitted herewith, Applicants respectfully maintain that the specification provides sufficient description and guidance to enable the claimed methods. Accordingly, Applicants respectfully request that this rejection be withdrawn.

Applicants respectfully request consideration of the remarks above. The Examiner is invited to call the undersigned agent if there are any questions.

To the extent necessary, a petition for an extension of time under 37 C.F.R. 1.136 is hereby made. Please charge any shortage in fees due in connection with the filing of this paper, including extension of time fees, to Deposit Account 502624 and please credit any excess fees to such deposit account.

Respectfully submitted,

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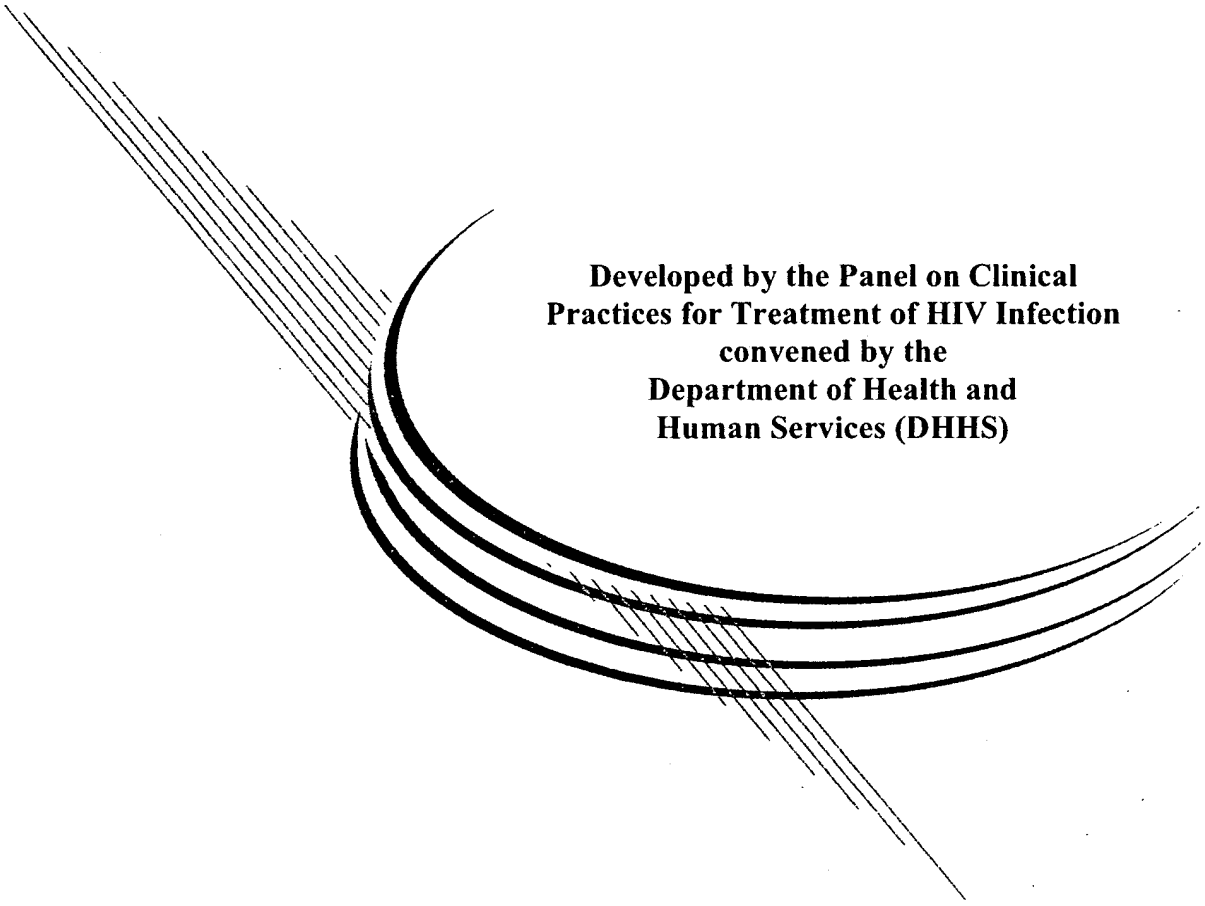
Please recognize our Customer No. 41552 as our correspondence address.

Guidelines for the Use of Antiretroviral Agents in HIV-1-Infected Adults and Adolescents

April 7, 2005



Developed by the Panel on Clinical Practices for Treatment of HIV Infection convened by the Department of Health and Human Services (DHHS)

It is emphasized that concepts relevant to HIV management evolve rapidly. The Panel has a mechanism to update recommendations on a regular basis, and the most recent information is available on the *AIDSinfo* Web site (http://*AIDSinfo*.nih.gov).

EXHIBIT A

April 7, 2005

Table of Contents

List of Tables and Figure

Guidelines for the Use of Antiretroviral Agents in HIV-1-Infected Adults and Adolescents

Introduction

Summary of Guidelines

Antiretroviral therapy for treatment of Human Immunodeficiency Virus type 1 (HIV-1) infection has improved steadily since the advent of combination therapy in 1996. More recently, new drugs have been approved, offering added dosing convenience and improved safety profiles, while some previously popular drugs are being used less often as their drawbacks become better defined. Resistance testing is used more commonly in clinical practice and interactions among antiretroviral agents and with other drugs have become more complex.

The Panel on Clinical Practices for Treatment of HIV (the Panel) develops these guidelines which outline current understanding of how clinicians should use antiretroviral drugs to treat adult and adolescents with HIV infections. The Panel considers new evidence and adjusts recommendations accordingly. The primary areas of attention and revision have included: when to initiate therapy, which drug combinations are preferred and which drugs or combinations should be avoided, and means to continue clinical benefit in the face of antiretroviral drug resistance. In contrast, some aspects of therapy, while important, have seen less rapid data evolution and thus fewer changes, such as medication adherence. Yet other topics have warranted more in-depth attention by separate guidelines groups, like the treatment of HIV during pregnancy.

Key Clinical Questions Addressed By Guidelines. For ease of use, these guidelines are organized so as to answer the following series of clinical questions clinicians are most likely to face in making treatment decisions:

- ***When should therapy be started in patients with established asymptomatic infection?*** The Panel reaffirms the desirability of initiating therapy before the CD4 cell count falls below 200 cells/mm^3. In addition, there are inconsistent data documenting added value in treating before the count falls below 350 cell/mm^3, but some clinicians opt to consider treatment in patients with CD4 count >350 cell/mm^3 and HIV-RNA >100,000 copies/mL. A review of the literature on this issue can been seen in the **When to Treat: Indications for Antiretroviral Therapy** section.
- ***Which regimens are preferred for initial therapy?*** The Panel continues to select several regimens as preferred, while appreciating that patient or provider preferences, or underlying co-morbidities, may make an alternative regimen better in such instances. The Panel recommends that an initial regimen contain two nucleoside/nucleotide reverse transcriptase inhibitors (NRTI) and either a non-nucleoside reverse transcriptase inhibitor (NNRTI) or a ritonavir-boosted or unboosted protease inhibitor (PI).
- ***What drugs or drug combinations should not be used?*** The Panel notes that certain drugs are so similar, for example, lamivudine and emtricitabine, that they should not be combined. Others have additive or synergistic toxicity, such as stavudine with didanosine, and should generally be avoided. Still others have intracellular interactions that decrease their antiviral activities, notably zidovudine with stavudine, and should thus be avoided.
- ***What are some limitations to the safety and efficacy of antiretroviral therapy?*** The Panel notes the high degree of medication adherence with all ARV regimens needed to prevent the selection of drug resistance. It also appreciates that short term and, even more concerning, longer term toxicity may limit the duration of treatment needed in what can be seen as a chronic disease. Finally, drug interactions among the antiretroviral drugs and with other necessary drugs are challenging and require special attention in prescribing and monitoring.
- ***What is the role of resistance testing in guiding therapy decisions?*** Resistance testing continues to be an important component of optimizing drug selection after treatment failure. However, its role in previously untreated persons is less clear. The Panel recognizes that there is a growing sense that such applications are of value, but little evidence exists to guide such use.

- ***What are the goals of therapy in treatment experienced patients?*** When possible, suppression of viremia to less than detection limits remains the goal of therapy. When this is not possible, the Panel recommends maintenance of even partial viremic suppression by selection of an optimal regimen based on resistance testing results. Either way, the ultimate goals are to prevent further immune deterioration and to avoid HIV-associated morbidity and mortality. The Panel recommends against complete antiretroviral cessation in late failure as this has resulted in rapid progression to AIDS and death.
- ***Are there special populations which may require specific considerations when using antiretroviral therapy?*** The Panel recognizes that there are subgroups of patients where specific considerations are critical when selecting and monitoring antiretroviral therapy, in order to assure safe and effective treatment. The Panel addresses some important antiretroviral related issues for these special populations, which include patients with acute HIV infection, HIV-infected adolescents, injection drug users, women of child bearing potential and pregnant women, and those with hepatitis B, hepatitis C, or tuberculosis co-infections.

Guidelines Process

These guidelines outline the current understanding of how clinicians should use antiretroviral agents to treat adults and adolescents infected with HIV-1. They were developed by the Panel on Clinical Practices for Treatment of HIV (the Panel), convened by DHHS.

Basis for Recommendations. Recommendations are based upon expert opinion and scientific evidence. Each recommendation has a letter/Roman numeral rating (**Table 1**). The letter indicates the strength of the recommendation based on the expert opinion of the Panel. The Roman numeral indicates the quality of the scientific evidence to support the recommendation. When appropriate data are not available, inconclusive, or contradictory, the recommendation is based on "expert opinion." These recommendations are not intended to supersede the judgment of clinicians who are knowledgeable in the care of HIV infection.

Updating of Guidelines. These guidelines generally represent the state of knowledge regarding the use of antiretroviral agents. However, as the science rapidly evolves, the availability of new agents and new clinical data may rapidly change therapeutic options and preferences. The guidelines are therefore updated frequently by the Panel, which meets monthly by teleconferencing to make ongoing revisions as necessary. All revisions are summarized and highlighted on the *AIDSinfo* Web site. Proposed revisions are posted for a public comment period, generally for 2 weeks, after which comments are reviewed by the Panel prior to finalization. Comments can be sent to **aidsinfowebmaster@aidsinfo.nih.gov**.

Other Guidelines. These guidelines focus on treatment for adults and adolescents. Separate guidelines outline how to use antiretroviral therapy for such populations as pregnant women, pediatric patients and health care workers with possible occupational exposure to HIV (see **http://aidsinfo.nih.gov/guidelines**). There is a brief discussion of the management of women in reproductive age and pregnant women in this document. However, for more detailed and up-to-date discussion on this and other special populations, the Panel defers to the designated expertise outlined by panels that have developed these guidelines.

Importance of HIV Expertise in Clinical Care. Multiple studies have demonstrated that better outcomes are achieved in patients cared for by a clinician with expertise *[1-6]*. This has been shown in terms of mortality, rate of hospitalizations, compliance with guidelines, cost of care, and adherence to medications. The definition of expertise in these studies has varied, but most rely on the number of patients actively managed. Based on this observation, the Panel recommends HIV primary care by a clinician with at least 20 HIV-infected patients and preferably at least 50 HIV-infected patients. Many authoritative groups have combined the recommendation based on active patients, along with fulfilling ongoing CME requirements on HIV-related topics.

BASIC EVALUATION

Pretreatment Evaluation

Each patient initially entering care should have a complete medical history, physical examination, and laboratory evaluation. The purpose is to confirm the presence of HIV infection, determine if HIV infection is acute (see **Acute HIV Infection**), determine the presence of co-infections, and assess overall health condition as recommended by the primary care guidelines for the management of HIV-infected patients *[7]*.

The following laboratory tests should be performed for each new patient during initial patient visits:

- HIV antibody testing (if laboratory confirmation not available) **(AI)**;
- CD4 cell count **(AI)**;
- Plasma HIV RNA **(AI)**;
- Complete blood count, chemistry profile, transaminase levels, BUN and creatinine, urinalysis, RPR or VDRL, tuberculin skin test (unless a history of prior tuberculosis or positive skin test), *Toxoplasma gondii* IgG, Hepatitis A, B, and C serologies, and PAP smear in women **(AIII)**;
- Fasting blood glucose and serum lipids if considered at risk for cardiovascular disease and for baseline evaluation prior to initiation of combination antiretroviral therapy **(AIII)**.

In addition:

- Resistance testing in chronically infected patients prior to initiating antiretroviral therapy is optional **(CIII)**;
- A test for *Chlamydia trachomatis* and *Neisseria gonorrhoeae* is optional **(BII)** in order to identify high risk behavior and the need for STD therapy;
- Chest x-ray if clinically indicated **(BIII)**.

Patients living with HIV infection must often cope with multiple social, psychiatric, and medical issues. Thus, the evaluation should also include assessment of substance abuse, economic factors, social support, mental illness, co-morbidities, and other factors that are known to impair the ability to adhere to treatment and to alter outcomes. Once evaluated, these factors should be managed accordingly.

Initial Assessment and Monitoring for Therapeutic Response

Two surrogate markers are routinely used to determine indications for treatment and to monitor the efficacy of therapy: $CD4^+$ T-cell count and plasma HIV RNA (or viral load).

$CD4^+$ T-cell count. The $CD4^+$ T-cell count (or CD4 count) serves as the major clinical indicator of immunocompetence in patients with HIV infection. It is usually the most important consideration in decisions to initiate antiretroviral therapy. The most recent CD4 cell count is the strongest predictor of subsequent disease progression and survival, according to clinical trials and cohort studies data on patients receiving antiretroviral therapy. A significant change between two tests (2 standard deviations) is defined as approximately 30% change of the absolute count and 3 percentage point change in CD4 percentage.

- **Use of CD4 for Initial Assessment**. The CD4 count is usually the most important consideration in decisions to initiate antiretroviral therapy. All patients should have a baseline CD4 cell count at entry into care **(AI)**; many authorities recommend two baseline measurements before decisions are made to initiate antiretroviral therapy due to wide variations in results **(CIII)**. The test should be repeated yet a third time if discordant results are seen **(AI)**. Recommendations for initiation of antiretroviral therapy based on CD4 cell count are found in the **When to Treat: Indications for Antiretroviral Therapy** section.
- **Use of CD4 Count for Monitoring Therapeutic Response**. Adequate viral suppression for most patients on therapy is defined as an increase in CD4 cell count that averages 100-150 cells/mm^3 per year with an accelerated response in the first three months. This is largely due to redistribution. Subsequent increases with good virologic control show an average increase of approximately 100 cells/mm^3 per year for the subsequent few years until a threshold is reached *[8]*.
- **Frequency of CD4 Count Monitoring.** In general, CD4 count should be determined every three to six months to (1) determine when to start antiretroviral in patients who do not meet the criteria for initiation; (2) assess immunologic response to antiretroviral therapy; and (3) assess the need for initiating chemoprophylaxis for opportunistic infections.

Viral Load. Plasma HIV RNA (viral load) may be a consideration in the decision to initiate therapy. In addition, viral load is critical for evaluating response to therapy **(AI)**. Three HIV viral load assays have been approved by the Food and Drug Administration (FDA) for clinical use:

- HIV-1 reverse transcriptase polymerase chain reaction assay (Amplicor HIV-1 Monitor Test, version 1.5, Roche Diagnostic);
- Nucleic acid amplification test for HIV RNA (NucliSens HIV-1 QT, Organon Teknika); and
- Signal amplification nucleic acid probe assay (VERSANT HIV-1RNA 3.0 assay, Bayer).

Analysis of 18 trials with over 5,000 participants with viral load monitoring showed a significant association between a decrease in plasma viremia and improved clinical outcome. Thus, viral load testing serves as a surrogate marker for treatment response and may be useful in predicting clinical progression. The minimal change in viral load considered to be statistically

significant (2 standard deviations) is a threefold or a 0.5 $\log_{10}$ copies/mL change. One key goal of therapy is a viral load below the limits of detection (at <50 copies/mL for the Amplicor assay, <75 copies/mL for the VERSANT assay, and <80 copies/mL for the NucliSens assay). This goal should be achieved by 16-24 weeks **(AI)**. Recommendations for the frequency of viral load monitoring are summarized below and in **Table 2**.

- **At Initiation or Change in Therapy**. Plasma viral load should be measured immediately before treatment, and at 2-8 weeks after treatment initiation or treatment changes due to suboptimal viral suppression. In the latter measure, there should be a decrease of at least a 1.0 $\log_{10}$ copies/mL **(BI)**.
- **In Patients With Viral Suppression Where Changes are Motivated by Drug Toxicity or Regimen Simplification**. Some experts also recommend repeating viral load measurement within 2-8 weeks after changing therapy. The purpose of viral load monitoring at this point is to confirm potency of the new regimen.**(BII)**
- **In Patients on a Stable Antiretroviral Regimen** The viral load testing should be repeated every 3-4 months thereafter or if clinically indicated.**(BII)** The testing should be repeated every 3-4 months thereafter or if clinically indicated. (**Table 2**)

Monitoring in Patients With Suboptimal Response. In addition to viral load monitoring, a number of additional factors should be assessed, such as non-adherence, altered pharmacology, or drug interactions. Resistance testing may be helpful in identifying the presence of resistance mutations that may necessitate a change in therapy. **(AII)**

TREATMENT GOALS

Eradication of HIV infection cannot be achieved with available antiretroviral regimens. This is chiefly because the pool of latently infected $CD4^+$ T cells is established during the earliest stages of acute HIV infection *[9]* and persists with a long half-life, even with prolonged suppression of plasma viremia *[10-13]*. Therefore, once the decision is made to initiate therapy, the primary goals of antiretroviral therapy are to:

- reduce HIV-related morbidity and mortality,
- improve quality of life,
- restore and preserve immunologic function, and
- maximally and durably suppress viral load.

Adoption of treatment strategies recommended in these guidelines has resulted in substantial reductions in HIV-related morbidity and mortality *[14-16]*.

Plasma viremia is a strong prognostic indicator of HIV disease progression *[17]*. Reductions in plasma viremia achieved with antiretroviral therapy account for substantial clinical benefits *[18]*. Therefore, suppression of plasma viremia as much as possible for as long as possible is a critical goal of antiretroviral therapy (see **Basic Evaluation: Initial Assessment and Monitoring for Therapeutic Response**). This goal, however, must be balanced against the need to preserve effective treatment options in patients who do not achieve undetectable viral load due to extensive viral resistance or persistent medication non-adherence.

Viral load reduction to below limits of assay detection in a treatment-naïve patient usually occurs within the first 16-24 weeks of therapy. However, maintenance of excellent treatment response is highly variable. Predictors of long-term virologic success include:

- potency of antiretroviral regimen,
- adherence to treatment regimen *[19, 20]*,
- low baseline viremia,
- higher baseline $CD4^+$ cell count *[19, 20]*, and
- rapid (i.e. $\geq$1 $\log_{10}$ in 1-4 months) reduction of viremia in response to treatment *[20]*.

Successful outcomes have not been observed across all patient populations, however. Studies have shown that approximately 70% of patients in urban clinic settings achieve the goal of no detectable virus compared to 80-90% in many clinical trials *[21]*.

Strategies to Achieve Treatment Goals

Achieving treatment goals requires a balance of sometimes competing considerations, outlined below. Providers and patients must work together to define priorities and determine treatment goals and options.

Selection of Combination Regimen. Several preferred and alternative antiretroviral regimens are recommended for use (see **What to Start With: Initial Combination Regimens for the Antiretroviral-Naïve Patient**). They vary in efficacy, pill burden, and potential side effects. A regimen tailored to the patient may be more successful in fully suppressing the virus with fewer side effects. Individual tailoring is based on such considerations as lifestyle, co-morbidities, and interactions with other medications.

Preservation of Future Treatment Options. Multiple changes in antiretroviral regimens, prompted by virologic failure due to drug resistant virus or patient non-adherence, can rapidly exhaust treatment options. While these are valid reasons to prompt a change in therapy, they should be considered carefully (see **Managing the Treatment Experienced Patient: Assessment of Antiretroviral Treatment Failure and Changing Therapy**).

Drug Sequencing. Appropriate sequencing of drugs for use in initial and subsequent salvage therapy preserves future treatment options and is another tool to maximize benefit from antiretroviral therapy. Currently recommended strategies spare at least two classes of drugs for later use and potentially avoid or delay certain class-specific side effects.

Improving Adherence. The reasons for variability in response to antiretrovirals are complex but may include inadequate adherence due to multiple social issues that confront patients *[22-24]*. Patient factors clearly associated with the risk of decreased adherence—such as active substance abuse, depression, and lack of social support—need to be addressed with patients before initiation of antiretroviral therapy *[25, 26]*. Strategies to improve medication adherence can improve outcomes.

WHEN TO TREAT: Indications for Antiretroviral Therapy

Panel's Recommendations (Table 4):

- ***Antiretroviral therapy is recommended for all patients with history of an AIDS-defining illness or severe symptoms of HIV infection regardless of CD4$^+$ T cell count. (AI)***
- ***Antiretroviral therapy is also recommended for asymptomatic patients with <200 CD4$^+$ T cells/mm^3(AI)***
- ***Asymptomatic patients with CD4$^+$ T cell counts of 201–350 cells/mm^3 should be offered treatment. (BII)***
- ***For asymptomatic patients with CD4$^+$ T cell of >350 cells/mm^3 and plasma HIV RNA >100,000 copies/ml most experienced clinicians defer therapy but some clinicians may consider initiating treatment. (CII)***
- ***Therapy should be deferred for patients with CD4$^+$ T cell counts of >350 cells /mm^3 and plasma HIV RNA <100,000 copies/mL. (DII)***

The decision to begin therapy for the asymptomatic patient is complex and must be made in the setting of careful patient counseling and education.

Considerations of initiating antiretroviral therapy should be primarily based on the prognosis of disease-free survival as determined by baseline CD4$^+$ T cell count *[27-29]* (**Figure A**; and **Table 3a**, **3b**). Also important are baseline viral load *[27-29]*, readiness of the patient to begin therapy; and assessment of potential benefits and risks of initiating therapy for asymptomatic persons, including short-and long-term adverse drug effects; the likelihood, after counseling and education, of adherence to the prescribed treatment regimen.

Recommendations vary according to the CD4 count and viral load of the patient, as follows.

<200 CD4$^+$ T cell count, with AIDS-defining illness, or symptomatic. Randomized clinical trials provide strong evidence of improved survival and reduced disease progression by treating symptomatic patients and patients with <200 CD4$^+$ T cells/mm^3 *[30-33]*. Observational cohorts indicate a strong relationship between lower CD4$^+$ T cell counts and higher plasma HIV RNA levels in terms of risk for progression to AIDS for untreated persons and antiretroviral naïve patients beginning treatment. These data provide strong support for the conclusion that therapy should be initiated in patients with CD4$^+$ T cell count <200 cells/mm^3 (**Figure A** and **Table 3a**) **(AI)** *[27, 28]*.

200-350 CD4$^+$ T cell count, patient asymptomatic. The optimal time to initiate antiretroviral therapy among asymptomatic patients with CD4$^+$ T cell counts >200 cells/mm^3 is unknown. For these patients, the strength of the recommendation for therapy must balance other considerations, such as patient readiness for treatment and potential drug toxicities.

After considering available data in terms of the relative risk for progression to AIDS at certain CD4$^+$ T cell counts and viral loads, and the potential risks and benefits associated with initiating therapy, most specialists in this area believe that the evidence supports initiating therapy in asymptomatic HIV-infected persons with a CD4$^+$ T cell count of 200-350 cells/mm^3 **(BII)**.

There is a paucity of data from randomized, controlled trials concerning clinical endpoints (e.g., the development of AIDS-defining illnesses or death) for asymptomatic persons with >200 CD4$^+$ T cells/mm^3 to guide decisions on when to initiate therapy. Observational data from cohorts of HIV-infected persons provide some guidance to assist in risk assessment for disease progression.

One source of observational data comes from cohorts of untreated individuals with regular measurements of $CD4^+$ T cell counts and HIV RNA levels. **Table 3b** is taken from a report by the CASCADE Collaboration, composed of 20 cohorts in Europe and Australia *[29]*. The information in this table provides an estimate of the short-term (6-month) risk of AIDS progression according to $CD4^+$ T cell count, HIV RNA level, and age. These estimates can be considered in making the decision about whether to start antiretroviral therapy before the next clinic visit.

Another source of observational data is from cohorts that follow patients after the initiation of antiretroviral treatment. A pooled analysis of 13 cohorts from Europe and North America provide the most precise information on prognosis following the initiation of treatment *[28]*. These data indicate that $CD4^+$ T-cell count is a much more important prognostic indicator than viral load for those initiating therapy. In this study, risk of progression was also greater for those with a viral load >100,000, older patients, those infected through injecting drug use, and those with a previous diagnosis of AIDS. The following chart shows the risk of progression to AIDS or death after 3 years, according to $CD4^+$ T-cell count and HIV RNA level at the time antiretroviral therapy was initiated. These data are from a large subset of patients less than 50 years old and without a history of an AIDS-defining illness or injection drug use:

$CD4^+$ T cell count	3 yr-probability	
	VL $<10^5$	VL $>10^5$
0 - 49 cells/mm^3	16 %	20%
50 - 99 cells/mm^3	12 %	16%
100 - 199 cells/mm^3	9.3 %	12%
200 - 349 cells/mm^3	4.7 %	6.1%
$\geq$350 cells/mm^3	3.4 %	4.4%

These data provide strong support for the recommendation, based on observational cohort , that therapy should be initiated before the $CD4^+$ T cell count declines to <200 cells/mm^3. However, differences in risk for those with $CD4^+$ T cell counts between 200–350 and >350 cells/mm^3 are based on too few events, and too short a follow-up period, to make reliable statements about when treatment should be started.

While there are clear strengths to these observational data, there are also important limitations. Uncontrolled confounding factors could impact estimates in both studies. Furthermore, neither study provides direct evidence on the optimum $CD4^+$ T cell count to begin therapy. Such data will have to come from studies that follow patients who start therapy at different $CD4^+$ T-cell counts above 200 cells/mm^3 and compare them with a similar group of patients (e.g., with similar $CD4^+$ T cell count and HIV RNA level) who defer treatment. To completely balance the benefits and risks of therapy, follow-up will have to examine progression to AIDS, major toxicities, and death.

>350 $CD4^+$ T cell count, patient asymptomatic. There is little evidence on the benefit of initiating therapy in asymptomatic patients with $CD4^+$ T cell count > 350 cells/mm^3. Most clinicians would defer therapy.

- The deferred treatment approach is based on the recognition that robust immune reconstitution still occurs in the majority of patients who initiate treatment while $CD4^+$ T cell counts are in the 200–350 cells/mm^{3}range. Also, toxicity risks and adherence challenges generally outweigh the benefits of initiating therapy at $CD4^+$ T cell counts >350 cells/mm^3. In the deferred treatment approach, increased levels of plasma HIV RNA (i.e., >100,000 copies/mL) are an indication for monitoring of $CD4^+$ T cell counts and plasma HIV RNA levels at least every three months, but not necessarily for initiation of therapy. For patients with HIV RNA <100,000 copies/mL, therapy should be deferred **(DII)**.
- In the early treatment approach, asymptomatic patients with $CD4^+$ T cell counts >350 cells/mm^3 and levels of plasma HIV RNA >100,000 copies/mL would be treated because of the risk for immunologic deterioration and disease progression **(CII)**.

An estimate of the short term risk of AIDS progression may be useful in guiding clinicians and patients as they weigh the risks and benefits of initiating versus deferring therapy in this CD4 cell range. As cited above, **Table 3b** provides an analysis of data from the CASCADE Collaboration, demonstrating the risk of AIDS progression within 6 months for different strata of $CD4^+$ T cell count, viral load, and age. As seen in **Table 3b**, a 55 year old with a $CD4^+$ T cell count of 350 and a HIV viral load of 300,000 copies/ml has a 5% chance of progression to an AIDS-defining diagnosis in 6 months, compared with a 1.2% chance for a similar patient with a viral load of 3,000 copies/mL.

Benefits and Risks of Treatment

In addition to the risks of disease progression, the decision to initiate antiretroviral therapy also is influenced by an assessment of other potential risks and benefits associated with treatment. Potential benefits and risks of early ($CD4^+$ T cell counts >350

cells/mm^3) or deferred ($CD4^+$ T cell count 200-350 cells/mm^3) therapy initiation for the asymptomatic patient should be considered by the clinician and patient.

Potential Benefits of Deferred Therapy include:

- avoidance of treatment-related negative effects on quality of life and drug-related toxicities;
- preservation of treatment options;
- delay in development of drug resistance if there is incomplete viral suppression;
- more time for the patient to have a greater understanding of treatment demands;
- decreased total time on medication with reduced chance of treatment fatigue; and
- more time for the development of more potent, less toxic, and better studied combinations of antiretrovirals.

Potential Risks of Deferred Therapy include:

- the possibility that damage to the immune system, which might otherwise be salvaged by earlier therapy, is irreversible;
- the increased possibility of progression to AIDS; and
- the increased risk for HIV transmission to others during a longer untreated period.

Gender Differences. The recommendation of when to start antiretroviral therapy is the same for HIV-infected adult male and female patients. Data regarding sex-specific differences in viral load and $CD4^+$ T cell counts are conflicting. Certain studies *[34-40]*, although not others *[41-44]*, have concluded that after adjustment for $CD4^+$ T cell counts, levels of HIV RNA are lower in women than in men. Although viral load is lower in women at seroconversion, the differences decrease with time, and the median viral load in women and men become similar within 5–6 years after seroconversion *[35, 36, 40]*. Importantly, rates of disease progression do not differ by gender *[38, 40, 45, 46]*. These data demonstrate that sex-based differences in viral load occur predominantly during a window of time when the $CD4^+$ T cell count is relatively preserved, when treatment is recommended only in the setting of increased levels of plasma HIV RNA.

Adherence Considerations. Concern about adherence to therapy is a major determinant for timing of initiation of therapy, with patient readiness to start treatment being a key factor in future adherence *[47]*. Depression and substance abuse may negatively impact adherence and response to therapy, therefore, should be addressed, whenever possible, prior to initiating therapy. However, no patient should automatically be excluded from consideration for antiretroviral therapy simply because he or she exhibits a behavior or other characteristic judged by the clinician to lend itself to non-adherence. Rather, the likelihood of patient adherence to a long-term drug regimen should be discussed and determined by the patient and clinician before therapy is initiated. To achieve the level of adherence necessary for effective therapy, providers are encouraged to use strategies for assessing and assisting adherence. (see **Adherence** section).

WHAT TO START WITH: Initial Combination Regimens for the Antiretroviral-Naïve Patient

Much progress has been made since zidovudine monotherapy demonstrated survival benefits in advanced HIV patients in the late 1980s *[48]*. As of October 2003, there were 20 approved antiretroviral agents, belonging to four classes, with which to design combination regimens containing at least three drugs. These four classes include the nucleoside/nucleotide reverse transcriptase inhibitors (NRTI), non-nucleoside reverse transcriptase inhibitors (NNRTI), protease inhibitors (PI), and fusion inhibitors (FI).

Summary of Recommended Regimens. Since the introduction in 1995 of PI and potent combination antiretroviral therapy (previously referred to as "highly active antiretroviral therapy" or "HAART"), a substantial body of clinical data has been amassed to guide the selection of initial therapy for the previously untreated patient. To date, most clinical experience with use of combination therapy in treatment-naïve individuals has been based on three different types of combination regimens, namely: NNRTI-based (1 NNRTI + 2 NRTI), PI-based (1-2 PI + 2 NRTI), and triple NRTI-based regimens. Recommendations are, accordingly, organized by these categories.

A list of Panel-recommended regimens for initial therapy in treatment naïve patients can be found in **Table 5**. In addition to notations in **Table 5**, **Criteria for Recommended Combination Antiretroviral Regimens** (below) outlines the rationale of the Panel's recommendations.

Potential advantages and disadvantages for each regimen recommended for initial therapy for treatment of naïve patients are listed in **Table 6** to guide prescribers in choosing the regimen best suited for an individual patient.

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HIV-specific immunity during structured antiviral drug treatment interruption☆

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Abstract

The immunologic correlates associated with control of viremia in HIV disease are poorly understood. We hypothesized that structured antiviral drug treatment interruptions could be utilized to better understand the relationship between HIV-specific immunity and viral replication. We thus examined the effects of two 8 weeks antiviral structured treatment interruptions (STIs) in a cohort of HIV-1 chronically infected individuals on highly active antiretroviral treatment (HAART) with ($n = 13$) and without ($n = 12$) therapeutic HIV immunizations. In this study, we observed that p24 gag antigen (np24) stimulated MIP-1β levels and T helper immune responses prior to antiviral drug discontinuation were associated with control of viremia. Stronger and earlier production of gag peptide stimulated gamma interferon was observed in the immunized group during the structured antiviral drug interruptions. These results support the concept that HIV-specific immune responses are associated with control of viremia. Further study of immune-based therapies that enhance HIV-specific immunity is warranted.
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Keywords: CD4; CD8; Beta chemokine

1. Introduction

Structured antiviral drug treatment interruptions (STIs) have been recently studied in an attempt to better understand the role of the immune system in HIV infection [1,2]. In primary HIV-1 or SIV infection, the preservation of HIV-specific T helper immune responses, measured as in vitro lymphocyte proliferative responses (LPR), has been observed with early antiviral drug initiation [3–5] and has also been associated with short term control of plasma viremia [6,7]. Most notably, in these studies strong HIV-1-specific T helper immune responses, similar to those observed in clinical non-progressors [3,5,8] have been observed in studies of primary HIV-1 infection with early HAART, prior to antiviral treatment interruption. Furthermore, in most studies of STIs, patterns of better virologic control were observed with additional cycles of interruptions compared to a single cycle [6,7].

Most trials of structured antiviral treatment interruption in chronic HIV have examined multiple STIs in subjects without pre-existing T helper immune responses [9,10]. We examined the effects of two 8 weeks antiviral treatment interruptions in a cohort of HIV-1 chronically infected individuals on HAART who had received therapeutic HIV immunization (Remune®) and had developed strong T helper immune responses prior to discontinuation of antiviral drug therapy ($n = 13$). We also examined the effects of two similar 8 week antiviral drug treatment interruptions in unimmunized, chronically infected individuals on HAART ($n = 12$). The goal of such a study was to examine the contribution of HIV-specific therapeutic immunization and surrogate markers in subjects while off antiviral drug therapy. Structured treatment interruptions were thus utilized to better understand the relationship between immunologic competence and viral replication. In this study, we observed that HIV-specific LPRs, interferon gamma by ELISPOT, and MIP-1β chemokine levels prior to the treatment interruptions correlated with the plasma viral load levels and CD4 counts

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EXHIBIT B

in chronically HIV infected subjects off antiviral drug therapy during the second but not the first STI. Stronger and earlier HIV-specific gag peptide stimulated gamma interferon production was observed in the immunized group. These results support the concept that strong HIV-specific immune responses are associated with control of viremia. Further study is warranted of immune-based therapies that enhance HIV-specific immunity to determine their clinical impact.

2. Methods

Chronically infected HIV-1 seropositive individuals (ELISA and Western blot) with CD4 cells counts >300 and plasma viral loads (HIV-1 RNA) of <2000 copies/ml were enrolled at a single site (San Diego Naval Medical Center) in an open label research study after institutional board approval and informed consent was obtained. Subjects were enrolled in an open label study if they met inclusion criteria and had a stimulation index to p24 antigen >10 and had participated in previous immunization protocols. All previously immunized subjects who were screened met the enrollment criteria. Unimmunized subjects were also enrolled if they met the enrollment criteria regardless of in vitro immune measurements. Subjects underwent two structured antiviral drug discontinuation periods for up to 8 weeks. Antiviral drug therapy was re-initiated for three consecutive HIV plasma RNA measurements of greater than 20,000 copies/ml or based on clinical parameters such as declines in CD4 cell counts. After the second STI, if viral load was less than 5000 copies/ml, subjects were given the option to remain off antiviral therapy or to re-initiate HAART and proceed to a third cycle of STI. Immunized subjects continued to receive Remune® every 12 weeks. CD4 cell counts and HIV-1 plasma RNA were obtained at screening, and weeks 0–8, 12, and 16–24. Plasma RNA was performed using the Amplicor ultrasensitive assay with a lower limit of quantification of 50 copies/ml (Hoffman La Roche, Nutley, NJ).

The HIV-1 immunogen (Remune®) consists of gp120-depleted, inactivated HIV-1 at a dose of 10 units of p24 antigen (100 μg total protein) in Incomplete Freund's Adjuvant (IFA). Gp120-depleted HIV-1 (HZ321) was also utilized for in vitro lymphocyte proliferation (LP) assays. Gp120-depleted HIV-1 immunogen was highly purified by ultrafiltration and ion exchange chromatography [11] from the filtered (0.45 μm) extracellular supernatant fluid of HZ321 Hut-78 cells [12,13]. HIV-1 antigen was clade A envelope and clade G gag [14]. The outer envelope protein (gp120) was depleted at the ultrafiltration stage of the purification process. Antigen preparations were inactivated through a sequential application of beta-propiolactone (BPL) [15] and ^{60}Co irradiation [16]. Native p24 was isolated from purified, inactivated HIV-1 treated with 2% Triton X-100 and then purified using Pharmacia Sepharose Fast Flow S resin. Chromatography was carried out at pH 5.0 and p24 was eluted using a linear salt gradient. Purity of the final product was estimated by both SDS-PAGE and reverse phase HPLC to be >99% with no immunoreactivity as measured by antibody reactivity to Classes I or II. PHA was obtained from Sigma (St. Louis, MO). Candida antigen was obtained from Greer laboratories (Lenoir, NC). Recombinant p24 antigen was obtained from Protein Sciences (Meridan, CT).

For the lymphocyte proliferation assays (LPA), fresh PBMCs from HIV-1 seropositive subjects were purified and cultured with medium alone or with inactivated HIV-1 gag antigens, including whole, gp120-depleted HIV-1 (5 μg/ml), np24 (5 μg/ml), rp24 (5 μg/ml), Candida (5 μg/ml), or PHA (5 μg/ml). PBMCs were seeded in a round bottom 96 well plate (Falcon) at 2×10^5 cells per well in complete RPMI medium (Hyclone) containing 10% heat-inactivated (30 min at 56 °C) human AB serum (Gemini), 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco), and L-glutamine 1% (Hyclone). All assays were performed in triplicate. After 6 days of incubation, cells were labeled with 1 μCi/well of ^{3}H-thymidine in complete RPMI for 16–24 h. On day 7, 20 μl of beta-propiolactone (1:1600 final concentration) was added to each well to inactivate any virus produced during the incubation period. Cells were harvested after a 2-h incubation in BPL at 37 °C and cells that incorporated label were determined by scintillation counting in a beta-counter. Geometric mean counts per minute (cpm) were calculated from the triplicate wells with and without antigen. Results were calculated as a lymphocyte stimulation index (LSI), which is the geometric mean cpm of the cells incubated with antigen divided by the geometric mean cpm of the cells without antigen (cells incubated in media alone).

All chemokine assays were performed in duplicate. MIP-1β quantification was determined by commercial ELISA from R&D Systems. MIP-1β measurements from frozen supernatants from antigen activated PBMCs were run in batch. MIP-1β quantification for specific antigens subtracted out no antigen (media alone) control wells.

ELISPOT assays were performed to measure the frequency of IFN-γ secreting cells on cryopreserved PBMCs. On day 1, a 96 well plate with a nitrocellulose-like bottom was coated with a capture monoclonal antibody. The plate (Millipore 0.45 μm Imobilon-P 96 well plate, catalog no. MAIPS4510, Millipore, Bedford, MA) was coated with 100 μl per well of a 2.5 μg/ml monoclonal anti IFN-γ (catalog no. M-700A, Endogen, Woburn, MA) in PBS and stored overnight at 4 °C. On day 2, after the plate was incubated overnight, the plate was washed six times with PBS. The plate was then blocked with RPMI media + 10% human serum for 1 h at 37 °C. After blocking, the plate was washed six times with PBS. In each well, 2×10^5 PBMC cells were tested in triplicate versus 5 μg/ml of the following test antigens; inactivated HIV-1 antigen, highly purified native p24 antigen (both from the Immune Response Corp., Carlsbad, CA), PHA (catalog L-9132, Sigma, St. Louis, MO); 2.5 μg/ml of each peptide from HIV-1 gag peptide pool consisting of the complete gag sequence (NIH reagent

program catalog no. 3992, clade B); and cells with media only as controls. On day 3, after the plate with cells was incubated for 24 h at 37 °C, it was washed six times with PBS. To detect IFN-γ bound to the plate, 100 μl of a 0.25 μg/ml biotinylated monoclonal anti IFN-γ (catalog no. M-701B, Endogen) in PBS was added to each well and incubated for 90 min at room temperature. The plate was washed six times with PBS + 0.05% Tween 20 and 100 μl of a 1:2000 dilution of streptavidin–alkaline phosphatase (catalog A-7294, Sigma) in PBS + 1% BSA was added to each well. The plate was incubated for 1 h at room temperature. The plate was then washed six times with PBS + 0.05% Tween 20. The 100 μl of the substrate BCIP/NBT (one tablet into 10 ml H_2O, catalog B-5655, Sigma) was then added to each well. The assay was stopped after spots emerged (5 min to 1 h) by washing with H_2O three times. The spots were counted using a dissection microscope (40×) with a Highlight 3000 light source (Olympus, Lake Success, NY). True spots were identified as having a dark center and a fading color intensity towards the edges. False spots are small, homogeneous in nature and have sharply defined borders. Interobserver correlation in counting was adequate ($r = 0.79$, $P = 0.0004$) as reported elsewhere [17]. Non-parametric Mann–Whitney U and Spearman Rank tests were utilized as the data was not normally distributed. Chi-square tests were used for categorical comparisons. All tests are two tailed.

3. Results

The baseline viral loads, CD4 cell counts, LPR, IFN-gamma, and MIP-1β levels on antiviral drug therapy are shown in Table 1 for the immunized and unimmunized groups. The immunized group received a median of nine immunizations prior to drug discontinuation. For the immunized group, HIV antigen stimulated LPR (median LSI pre-immunization = 5.26; median LSI post-immunization = 53.01, $P = 0.0006$) and native p24 antigen stimulated LPR (median LSI pre-immunization = 2.28; median LSI post-immunization = 31.48, $P < 0.0001$) increased after immunization and prior to drug discontinuation. Similarly, for the immunized group, HIV antigen stimulated MIP-1β (median MIP-1β pre-immunization = 420 pg/ml; median MIP-1β post-immunization = 5814 pg/ml, $P = 0.0008$) and native p24 antigen stimulated MIP-1β (median MIP-1β pre-immunization = 144 pg/ml; median MIP-1β post-immunization = 2502 pg/ml, $P = 0.007$) were significantly increased after immunization and prior to drug discontinuation. The median days on antiviral drugs prior to treatment interruption was 1534 days for the immunized group and 1304 for the unimmunized group. As shown in Table 1, prior to HAART interruption there was no significant difference between the immunized and unimmunized groups for CD4 cell counts, HIV-1 RNA, or pooled gag peptide stimulated gamma interferon production by ELISPOT, but the two groups were significantly different for HIV ($P = 0.002$) and native p24 ($P = 0.0006$) LPRs. HIV ($P = 0.004$) stimulated gamma interferon production by ELISPOT and HIV ($P = 0.0003$) and native p24 ($P = 0.02$) stimulated MIP-1β production.

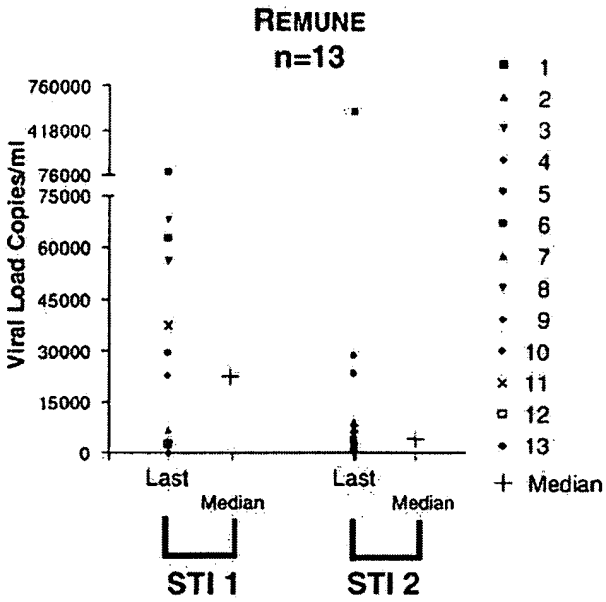

Fig. 1. Viral load for the immunized group during the first and second STIs.

The immunized group ($n = 13$) had a final median VL of 22,570 copies/ml during the first structured antiviral drug treatment interruption (Fig. 1). During the second

Table 1
Viral load, CD4, LPR, MIP-1β, and gamma interferon production for the immunized and unimmunized group prior to drug discontinuation

	Median baseline CD4 (cells/μl)	Median baseline viral load (copies/ml)	Median baseline						
			Median baseline (SI)		MIP-1β (pg/ml)		(IFN-γ)/2 × 10^5 PBMC		
			HIV LP	np24 LP	HIV	np24	HIV	np24	Peptide
Remune ($n = 13$)	751	49	53.01**	31.48***	5814***	2502*	3.67**	1.33	11.33
Un-immunized ($n = 12$)	666	49	15.23	4.63	93	524[a]	0	0	6.33

[a] $n = 11$ for this value.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

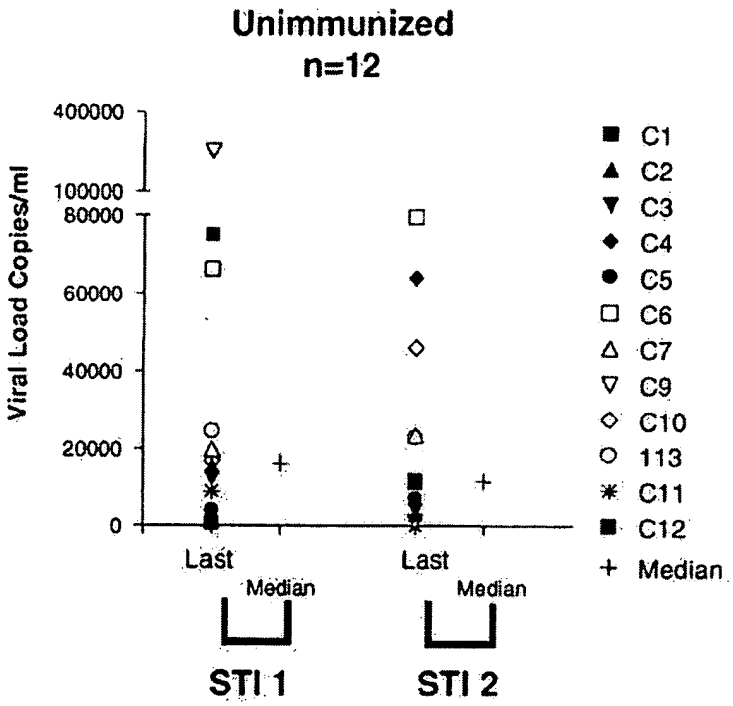

Fig. 2. Viral load for the unimmunized group during the first and second STIs.

interruption, the final median viral load was 4247 copies/ml. The percent of subjects in the immunized group below 5000 copies at the end of the first STI was 38 (5/13) and 53% (7/13) during the second STI. There was a trend toward an increased number of subjects with a viral load of less than 10,000 copies at the end the second STI 77% (10/13) compared to the end of the first STI 46% (6/13) ($P = 0.10$) in the immunized group. For the immunized group CD4+ cell counts were not significantly ($P > 0.05$) different after one (median = 761 cells/μl) or two interruptions (median = 945 cells/μl).

The unimmunized group ($n = 12$) had a final median VL of 16,110 copies/ml during the first interruption (Fig. 2). During the second interruption the final median VL was 11,480 copies/ml. The percent of subjects with less than 5000 copies/ml at the end of the first STI was 17 (2/12) and 33% (4/12) at the end of the second STI in the unimmunized group. The percent of subjects with less than 10,000 copies/ml was 25% (3/12) at the end of the first STI and 41% (5/12) at the end of the second STI. Similarly, there were no differences in CD4 cell counts after one (median = 603 cells/μl) or two interruptions (median = 544 cells/μl) in the unimmunized group.

We also examined the effect of drug interruptions on the HIV and native p24 antigen stimulated lymphocyte proliferative response and MIP-1β levels for the immunized and non-immunized groups. The immunized group maintained their strong LPR and MIP-1β production to both HIV and native p24 protein antigen during the two cycles of antiviral drug interruption (data not shown). The unimmunized group maintained a low LPR to HIV (data not shown) and p24 antigen during the two cycles of drug interruption. Furthermore, the unimmunized group maintained a low level of native p24 and HIV protein antigen stimulated MIP-1β production (data not shown). However, gamma interferon production in response to pooled gag peptide was significantly increased from baseline in the immunized group (Fig. 3a) during the first ($P = 0.04$) and second STI ($P = 0.02$) but not in the unimmunized group (Fig. 3b). Furthermore, the gamma interferon response as measured by ELISPOT to HIV proteins correlated with pooled gag peptides response in the immunized group at baseline (HIV: $r = 0.65$, $P = 0.02$; p24: $r = 0.60$, $P = 0.03$), and during the first STI (HIV: $r = 0.72$, $P = 0.006$; p24: $r = 0.75$, $P = 0.003$) and second STI (HIV: $r = 0.52$, $P = 0.07$; p24: $r = 0.53$, $P = 0.06$). In contrast, the protein and peptide ELISPOT responses did not correlate in the unimmunized group.

Finally, we examined whether the cell-mediated immune response prior to drug interruption correlated with virus or CD4 cell counts while off therapy for both the immunized and unimmunized subjects. As shown in Table 2, HIV antigen specific LPR, gamma interferon production by ELISPOT, and np24 MIP-1β levels prior to drug interruption correlated negatively with the last viral load and positively with CD4 cell counts during the second interruption (data not shown). No such association was observed during the first STI. By the LPR assay, response to PHA or Candida antigen prior to treatment discontinuation did not demonstrate a similar association (data not shown). The

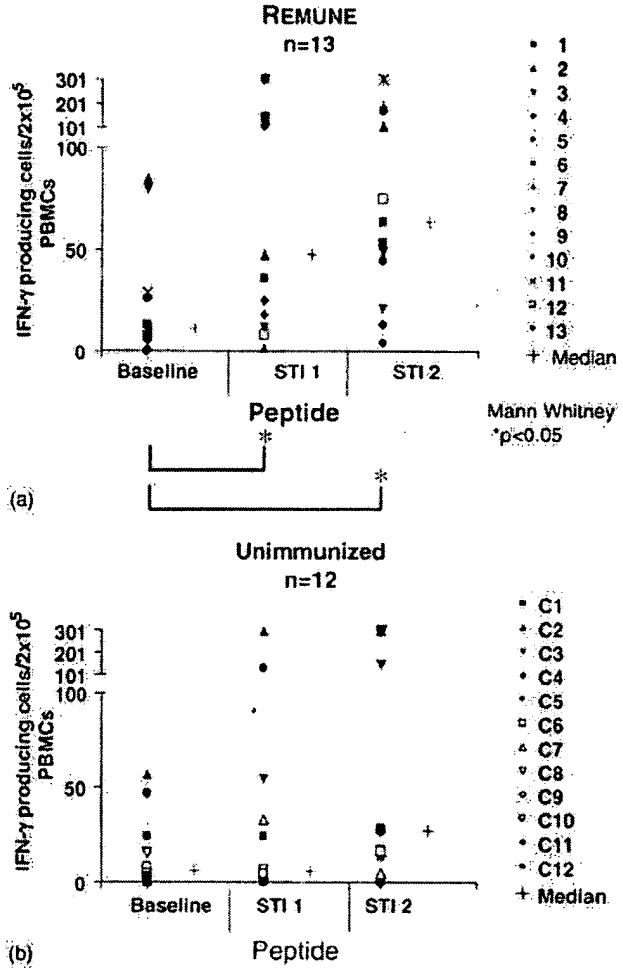

Fig. 3. (a) Interferon by ELISPOT to gag peptide pool during one and two cycles of STI ($n = 13$) in the immunized group. Each symbol represents a different individual. Crosses represent medians; (b) interferon by ELISPOT to gag peptide pool during one and two cycles of STI ($n = 12$) in the unimmunized group. Each symbol represents a different individual. Crosses represent medians.

Table 2
Correlations between HIV-specific protein stimulated LPR, MIP-1β, and gamma interferon production prior to drug discontinuation and viral load peak, last viral load and last CD4 cell count during the second STI

Baseline	Second STI			
	Last VL		Last CD4#	
	r	P	r	P
HIV LPR	−0.41	0.04	0.34	0.09
np24 LPR	−0.32	0.11	0.36	0.07
HIV MIP-1β	−0.32	0.12	0.28	0.18
np24 MIP-1β	−0.56	0.005	0.41	0.05
HIV (IFN-γ)/2 × 10^5 PBMCs	−0.36	0.08	0.46	0.02
np24 (IFN-γ)/2 × 10^5 PBMCs	−0.41	0.04	0.42	0.03

strongest correlation was noted to be between p24 antigen stimulated MIP-1β prior to antiviral drug discontinuation and the last viral load during the second drug interruption ($r = -0.56$, $P = 0.005$).

4. Discussion

In this study, we examined the relationship between immune responses and viral replication in subjects who underwent two cycles of treatment interruptions. Of note, the immunized group had augmented HIV-specific T helper immune responses and β-chemokines after immunization and prior to antiviral drug treatment discontinuation. These type of immune responses may play different roles as the T helper immune response may be thought to orchestrate an effect on the CD8+ T cell response [18]. β-Chemokines are factors that can be produced by a variety of cell types and are thought to bind the CCR 5 co-receptors for HIV-1, thereby impacting on viral load. This study confirms previous observations that both T helper immune responses and β-chemokines can be stimulated with therapeutic vaccination, and previous studies suggest that these increases in HIV-1-specific immunity are not observed when immunization consists of the adjuvant alone [19] (F. Valentine and E. Fernandez-Cruz, personal communications).

In this study, we attempted to determine whether or not HIV-specific immune responses prior to antiviral drug treatment interruption were related to viral load or CD4 cell counts off therapy. The results suggest that both T helper immune responses, measured as lymphocyte proliferation or ELISPOT and in vitro MIP-1β chemokine levels correlated with control of viremia and with CD4 cell counts of antiviral drug therapy. This correlation became apparent during the second but not first drug discontinuation. Thus, we speculate that a single, brief drug interruption may be inadequate to determine whether immune mechanisms are involved in the control viral replication. It is possible that viral replication during the first STI may activate CD8+ T cells, as viral replication peaks. These cells can then act on controlling viral replication more efficiently in the presence of adequate HIV-specific CD4+ T helper cells and β-chemokines, as observed during the second STI.

Such a hypothesis is supported by the observation that an enhancement of gag peptide stimulated production of gamma interferon was observed in the immunized group but not unimmunized group during the STIs. In addition, protein and peptide responses correlated in the immunized but not unimmunized group. Work by other groups [20] has demonstrated that immune responses to HIV peptides and proteins are primarily CD8+ and CD4+ T cell responses, respectively. The implication of these findings is that stronger T helper immune responses prior to treatment interruption may play a role in stimulating stronger peptide induced CD8+ T cell responses during the STIs. Longer periods of cycles of

treatment interruptions are thus warranted in order to clarify the relationship between CD8+ T cell responses and control of viral replication.

In summary, this study suggests that in vitro HIV-specific immune responses prior to antiviral drug discontinuation correlate with control of viremia and CD4+ T cell counts in HIV-1 infected subjects off therapy. The np24 antigen stimulated MIP-1β levels prior to antiviral drug discontinuation were strongly correlated with control of viral load and increased CD4 cell counts. In addition, HIV-specific gag peptide stimulated production of gamma interferon was augmented in the immunized but not unimmunized group during the STIs. These results suggest that larger and longer studies are warranted to further examine the relationship between HIV-specific immune function and control of viremia. This study further suggests that immune-based therapies that enhance HIV-specific immunity should be studied to determine whether they result in improving clinical outcomes for subjects with chronic HIV-1 infection.

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Loss of HIV-1–specific CD8$^+$ T Cell Proliferation after Acute HIV-1 Infection and Restoration by Vaccine-induced HIV-1–specific CD4$^+$ T Cells

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Abstract

Virus-specific CD8$^+$ T cells are associated with declining viremia in acute human immunodeficiency virus (HIV)1 infection, but do not correlate with control of viremia in chronic infection, suggesting a progressive functional defect not measured by interferon γ assays presently used. Here, we demonstrate that HIV-1–specific CD8$^+$ T cells proliferate rapidly upon encounter with cognate antigen in acute infection, but lose this capacity with ongoing viral replication. This functional defect can be induced in vitro by depletion of CD4$^+$ T cells or addition of interleukin 2–neutralizing antibodies, and can be corrected in chronic infection in vitro by addition of autologous CD4$^+$ T cells isolated during acute infection and in vivo by vaccine-mediated induction of HIV-1–specific CD4$^+$ T helper cell responses. These data demonstrate a loss of HIV-1–specific CD8$^+$ T cell function that not only correlates with progressive infection, but also can be restored in chronic infection by augmentation of HIV-1–specific T helper cell function. This identification of a reversible defect in cell-mediated immunity in chronic HIV-1 infection has important implications for immunotherapeutic interventions.

Key words: HIV-1 • CD8$^+$ T cells • CD4$^+$ T cells • vaccine • protective immunity

Introduction

Acute HIV-1 infection is characterized by high level plasma viremia, which leads to an activation of the cellular immune system and the rapid expansion of HIV-1–specific CD8$^+$ T cells (1). The first appearance of these cells in the peripheral blood is followed by a rapid and dramatic decline of HIV-1 plasma viremia, probably reflecting the strong antiviral activities of these cells (2, 3). The HIV-1–specific CD8$^+$ T cell responses detected during acute HIV-1 infection are typically low in magnitude and narrowly directed against a paucity of viral epitopes (4–6). Thus, the apparent antiviral activity of these cells in acute infection constitutes a striking contrast to chronic HIV-1 infection, where high levels of viral replication occur in the presence of strong, polyclonal and broadly diversified HIV-1–specific CD8$^+$ T cell responses, as determined by the assessment of antigen-specific interferon γ secretion (7–9). These data suggest a progressive functional defect of HIV-1–specific CD8$^+$ T cells in chronic infection that is not measured by assays quantifying solely antigen-specific interferon γ production of T cells.

Recent data demonstrated that HIV-1–specific CD8$^+$ T cells in individuals with long-term nonprogressive infection have a strong HIV-1–specific ex vivo proliferative capacity, whereas this effector function seems to be absent in individuals with high level viremia (10). A similar loss of HIV-1–specific ex vivo proliferation was also observed for HIV-1–specific CD4$^+$ T cells, which show strong proliferative capacities in

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Abbreviations used in this paper: CFSE, carboxyfluorescein succinimidyl ester; FSC, forward scatter; SFC, spot-forming cell; SSC, side scatter.

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acute and long-term nonprogressive infection (11–13), but no detectable ex vivo proliferation in the presence of ongoing viral replication. However, a direct functional connection between these two cell subsets has not been revealed and functional interactions between HIV-1–specific $CD4^+$ and $CD8^+$ T cells are currently insufficiently understood.

In this study, we demonstrate that HIV-1–specific $CD4^+$ and $CD8^+$ T cells in acute HIV-1 infection have strong ex vivo proliferative capacities, which are rapidly lost in the presence of continuing viral replication, but partially preserved by early institution of antiretroviral therapy. HIV-1–specific proliferation of $CD8^+$ T cells critically depended on the presence of IL-2–secreting antigen-specific $CD4^+$ T cells, as it was restored in $CD8^+$ T cells from the phase of chronic infection in vitro by the addition of autologous HIV-specific $CD4^+$ T cells isolated during acute infection and more importantly, in vivo by the induction of HIV-1–specific $CD4^+$ T helper cell responses using an HIV-1 immunogen. Overall, these data suggest that the proliferative impairment of HIV-1–specific $CD8^+$ T cells during chronic infection is not primarily due to an intrinsic functional defect of these cells, but rather represents a direct consequence of the progressive loss of IL-2–secreting, HIV-1–specific $CD4^+$ T cells.

Materials and Methods

Study Individuals. A total of 35 HIV-1–infected individuals recruited from the Massachusetts General Hospital, the Fenway Community Health Care Center, or the Lemuel Shattuck Hospital were included in this study. 18 persons had primary HIV-1 infection, defined by negative or incompletely positive HIV-1–specific ELISA and/or Western blot reactions in the presence of detectable viral load or HIV-1 seroconversion within 6 mo before study enrollment (4). Seven additional subjects with long-term nonprogressive disease courses ($CD4^+$ T cell count of >500/ml and viral load of <1,000 copies/ml for at least 5 yr in the absence of antiretroviral therapy) and 10 individuals with chronic progressive HIV-1 infection (viral load of >30,000 copies/ml or $CD4^+$ T cell count of <300 cell/μl) were also included. The clinical and demographic characteristics of the study individuals are summarized in Table I. In addition, cryopreserved PBMCs from 10 study individuals who had previously participated in a clinical pilot trial using an HIV-1 immunogen (14) were analyzed in this study. The study was approved by the respective institutional review boards and was conducted in accordance with human experimentation guidelines of the Massachusetts General Hospital.

HLA Typing. High and intermediate resolution HLA class I typing was performed at a commercial laboratory (Dynal) by sequence-specific PCRs according to standard procedures. DNA for typing was extracted using the Purgene DNA Isolation kit for whole blood samples (Gentra Systems).

Lymphocyte Separation and Culture. Blood specimens were drawn in ACD tubes (Becton Dickinson). Fresh PBMCs were separated from whole blood by Ficoll-Hypaque (Histopaque 1077; Sigma-Aldrich) density gradient centrifugation. PBMCs were cultured in RPMI 1640 medium (Sigma-Aldrich) supplemented with 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, 10 mM Hepes, and 10% heat-inactivated FCS. Recombinant human IL-2 (provided by the AIDS Research & Reference Reagent Repository, National Institutes of Health) was added in some assays at a concentration of 50I U/ml.

Synthetic Peptides. 410 synthetic 17–19 amino acid peptides, overlapping by 10 amino acids and spanning the entire HIV-1 clade B 2001 consensus sequence (http://hiv-web.lanl.gov), and peptides corresponding to described optimal HIV-1 $CD8^+$ T cell epitopes (15), were synthesized at the Massachusetts General Hospital Peptide Core Facility on an automated peptide synthesizer using F-moc technology. 14 CMV-specific peptides that were selected on the basis of their capacity to bind to HLA class II DRB1*0401 in binding assays (16) were similarly synthesized.

ELISPOT Assay. ELISPOT assays were performed as described previously (4). In brief, PBMCs were plated in 96-well polyvinylidene plates that had been precoated with 0.5 μg/ml of an anti–human interferon γ mAb (Mabtech). PBMCs were added at a concentration of 50,000–100,000 cells per well in a volume of 100 μl RPMI 1640 medium supplemented with 10% FCS, 10 mM Hepes buffer, 2 mM L-glutamine, and 50 U/ml penicillin-streptomycin. The final concentration of the peptides in every single well was 14 μg/ml. Plates were incubated overnight at 37°C, 5% CO_2, and developed on the next day as described elsewhere (17). Wells containing PBMCs and medium with phytohemagglutinin or without any peptide were used as positive or negative controls and run in triplicate on each plate. To calculate the number of specific T cells, the number of spots in the negative control wells was subtracted from the counted number of spots in each well. Responses were considered positive if there were >50 spot-forming cells (SFCs)/10^6 PBMCs and at least three times the mean number of SFCs of the three control wells. $CD8^+$ T cell dependence of responses was determined by depletion of $CD4^+$ T cells using the MiniMACS cell depletion system (Miltenyi Biotec).

Ex Vivo Proliferation Assay. PBMCs were first suspended at 10^6/ml in PBS and incubated at 37°C for 7 min with 0.25 μM carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes). After the addition of serum and washes with PBS, cells were suspended at 10^6/ml in medium (RPMI 1640 supplemented with glutamine, 10% human FCS, penicillin, and streptomycin). No exogenous cytokines were added to the medium unless otherwise indicated. Pools of overlapping HIV-1–specific peptides representing the entire amino acid sequence of either Gag, Nef, Pol, Env, a combined pool of Tat, Rev, Vif, Vpr, Vpu, or tetanus toxoid (Aventis Behring) were then added at a concentration of 20 ng/ml per peptide. On day 6, cells were harvested, washed with PBS, and stained with mAbs (CD4 PE, CD8 APC, and CD3 PerCP; BD Biosciences). Cells were then washed and fixed in 1% paraformaldehyde and subjected to flow cytometric analysis. Where indicated, IL-2–neutralizing antibodies (clone MQ1-17H12; BD Biosciences) or isotype control antibodies (clone A4A; Neomarkers) were added at 10 μg/ml. In some experiments, 10^6 autologous $CD4^+$ T cells isolated and cryopreserved during acute or chronic HIV-1 infection were thawed and added to 5×10^6 PBMCs from chronic HIV-1 infection. Flow cytometric data (100,000 nongated events) were acquired on a FACSCalibur four-color flow cytometer using CELLQuest software or an LSR II flow cytometer using the FACS DiVa software (all instruments and software from BD Biosciences). The mean background proliferation was calculated based on the proliferating fractions in media alone. The antigen-specific proportion of proliferating cells was calculated by subtracting the proportion of proliferating cells in unstimulated samples from the proliferating fraction in response to antigen.

Multiparameter Flow Cytometric Analysis. We used a panel of five different monoclonal surface antibodies (CD25 [IL-2Rα chain] PE-Cy7, CD8 APC-Cy7, CD4 APC-Cy5.5, IL-7Rα

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chain PE, and IL-15Rα chain Alexa 430; all from BD Biosciences) in addition to staining with APC-labeled MHC class I–peptide tetramer complexes (Beckman Coulter) and CFSE. For intracellular cytokine stainings, cells were initially stained with surface antibodies. After fixation and permeabilization, cells were stained with intracellular antibodies as described previously (17). Samples were acquired on an LSR II flow cytometric device (BD Biosciences), using the FACS DiVa software (BD Biosciences) according to the manufacturer's instructions. Data analysis was performed with the FlowJo software package (TreeStar).

Depletion and Enrichment of Selective Cellular Subsets. Isolation of $CD8^+$ and $CD4^+$ T cells as well as depletion of $CD3^+$ T cells from whole blood samples was performed by use of the respective Rosette Sep cell separation kits (StemCell Technologies Inc.). Depletion of $CD4^+$ from isolated PBMCs was performed using magnetic anti-$CD4^+$ beads and the MACS cell separation system (Miltenyi Biotec). All cell enrichment and depletion procedures were conducted by negative selection to ensure that isolated cells were not labeled with antibodies.

Statistical Analysis. Results are given as means or medians with ranges. Statistical analysis was based on Student's *t* tests. A p-value of <0.05 was considered significant. When two adjacent peptides were recognized in the ELISPOT assays, we deleted the weaker of the two responses. In the case of three adjacent peptides eliciting a response, the weakest of all three peptides was deleted and the responses were counted as two epitopic regions. Statistical analysis and graphical presentation was performed by use of the GraphPad Prism software package.

Results

Strong Lymphoproliferative Responses of HIV-1–specific $CD8^+$ T Cells in Primary HIV-1 Infection. A number of recent studies have analyzed the magnitude, breadth, and protein specificity of HIV-1–specific $CD8^+$ T cell responses in primary HIV-1 infection by interferon γ ELISPOT technology or intracellular cytokine staining (4–6, 18). Here, we extended these studies and used a flow cytometric proliferation assay based on the sequential loss of CFSE labeling in dividing cells to determine the ex vivo proliferative capacity of HIV-1–specific $CD8^+$ T cells in a total of 18 different subjects with primary HIV-1 infection. In addition, 10 individuals with untreated chronic progressive HIV-1 infection and 7 individuals with untreated long-term nonprogressive HIV-1 infection were included as reference populations. The demographic and clinical characteristics of the study subjects are summarized in Table I.

Fig. 1 shows representative experimental results from individuals with long-term nonprogressive HIV-1 infection (Fig. 1 A), chronic progressive HIV-1 infection (Fig. 1 B), and primary HIV-1 infection (Fig. 1 C). Although similar frequencies of dividing $CD8^+$ T cells were observed in the three study subjects after stimulation with PHA, populations of proliferating antigen-specific $CD8^+$ T cells were only seen in subjects with primary or long-term nonprogressive HIV-1 infection after stimulation of cells with a pool of overlapping peptides spanning the HIV-1 Nef protein. These proliferating $CD8^+$ T cells were specific for HIV-1 Nef, as demonstrated by the specific staining with MHC class I tetramers refolded with HIV-1 Nef peptides (Fig. 1, D and E). Overall, the proportion of $CD8^+$ T cells proliferating in response to stimulation with viral peptides spanning the entire HIV-1 proteome reached a median of 10.9% (range: 3.5–22%) and 23.6% (range: 11.8–59.9%) in individuals with primary or long-term nonprogressing HIV-1 infection, respectively. In contrast, essentially no

Table I. *Demographical and Clinical Characteristics of the Study Persons*

Study population	Age (median, range)	Sex (m/f ratio)	Ethnicity	$CD4^+$ T cell count (median, range)		HIV-1 RNA (median, range)	
				Baseline	1 yr follow-up	Baseline	1 yr follow-up
Subjects with long-term nonprogressing HIV-1 infection (n = 7)	47 (34–51)	5:2	6 Caucasians, 1 Hispanic	805 cells/μl (566–1,118)	n/a	555 copies/ml (<50–1,000)	n/a
Subjects with primary HIV-1 infection (n = 18)	46.4 (32–57)	17:1	16 Caucasians, 1 African American, 1 Haitian	562 cells/μl (307–1,141)	*untreated:* 490 cells/μl (342–1,050) *treated:* 753 cells/μl (434–948)	105,000 copies/ml (216–2,500,000)	*untreated:* 26,300 copies/ml (4,231–63,900) *treated:* <50 copies/ml (all patients)
Subjects with chronic progressive HIV-1 infection (n = 10)	42.5 (37–56)	9:1	5 Caucasians, 2 African Americans, 2 Hispanics	177 cells/μl (20–460)	n/a	42,344 copies/ml (7,247–232,000)	n/a

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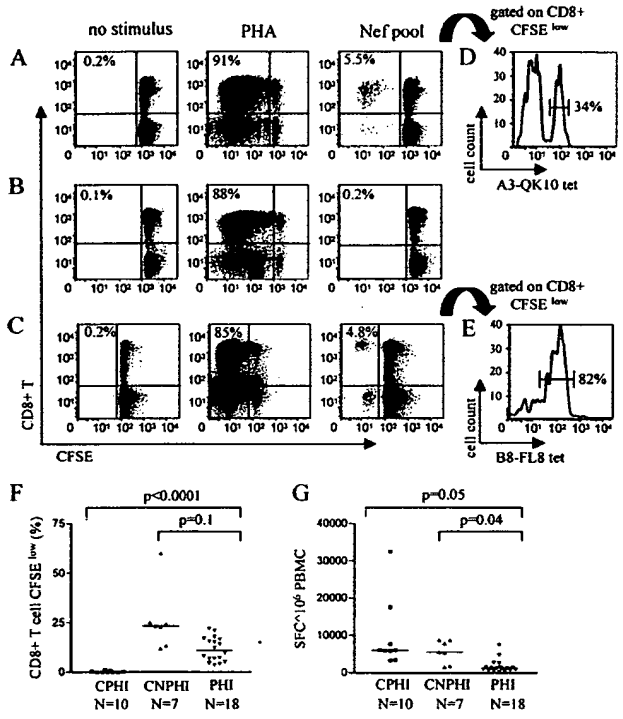

Figure 1. Cross-sectional assessment of CD8+ T cell proliferation after stimulation with HIV-1 peptide pools in individuals with primary, chronic progressive, and chronic long-term nonprogressive HIV-1 infection. (A–C) Dot plots showing the flow cytometric analysis of HIV-1–specific CD8+ T cell proliferation after stimulation of PBMCs with no stimulus, phytohemagglutinin (PHA), or a pool of overlapping peptides spanning the entire HIV-1 Nef protein in subjects with long-term nonprogressive (A), chronic progressive (B), or primary (C) HIV-1 infection. Values in top left corner of dot plots indicate the proportion of $CFSE^{low}$ CD8+ T cells. (D and E) Corresponding antigen specificity of proliferation cells. 34% of proliferating cells in the study individual in A were binding to the HLA-A3-QVPLRPMTYK (QK10) tetramer (D), whereas 82% of the CD8+ T cells proliferating in the study person in C were specific for the HLA-B8-FLKEKGGL (FL8) tetramer (E). (F and G) Comparative analysis of proliferation and interferon γ secretion by CD8+ T cells in response to stimulation with overlapping peptides spanning the entire HIV-1 proteome. Data from study subjects with chronic progressive HIV-1 infection (CPHI; n = 10), chronic long-term nonprogressive HIV-1 infection (CNPHI; n = 7), and primary HIV-1 infection (PHI; n = 18) are shown.

HIV-1–specific CD8+ T cell ex vivo proliferation was observed in study persons with chronic progressive HIV-1 infection (Fig. 1 F; reference 10), whereas the proportion of proliferating CMV-specific CD8+ T cells was not reduced in a subset of individuals with chronic HIV-1 infection when compared with persons with acute infection (median of 7% [range: 4–17%] vs. 9.1% [range: 7.5–23%]; P = 0.5).

The lymphoproliferative CD8+ T cell responses in primary HIV-1 infection targeted multiple viral regions, with a median proportion of 2.5% (range: 0–16.4%), 1.6% (range: 0–15%), 0.3% (range: 0–5.3%), 0.25% (range: 0–11%), or 0.1% (range: 0–6.48%) of CD8+ T cells responding to stimulation with Nef, Gag, Pol, Env, or the remaining regulatory and accessory HIV-1 proteins (Vpr, Vpu, Vif, Rev, and Tat), respectively. Notably, the observed pattern of lymphoproliferative HIV-1–specific CD8+ T cells in the three study groups was strikingly different from the corresponding HIV-1–specific CD8+ T cell responses measured using an interferon γ ELISPOT assay. As described previously (4, 7), the total magnitude of interferon γ–secreting, HIV-1–specific CD8+ T cells in individuals with primary HIV-1 infection was significantly lower than in individuals with chronic infection, whereas no difference in the total HIV-1–specific CD8+ T cell magnitude was found between individuals with progressive and long-term nonprogressive chronic infection (Fig. 1 G). Taken together, these data show that HIV-1–specific CD8+ T cells in primary and long-term nonprogressive HIV-1 infection have strong ex vivo proliferative capacities, whereas this effector function is absent in chronic progressive HIV-1 infection.

Parallel Evolution of Lymphoproliferative HIV-1–specific CD4+ and CD8+ T Cell Responses after Primary HIV-1 Infection. To more closely determine the fate of HIV-1–specific CD8+ T cell lymphoproliferative responses mounted during primary HIV-1 infection, we longitudinally followed the evolution of these responses during the ensuing disease process, using the CFSE-based proliferation assay in conjunction with an interferon γ ELISPOT assay. In line with previous findings (4, 19), we observed that the total magnitude of HIV-1–specific interferon γ–secreting CD8+ T cells increased by a median of threefold over a 1-yr period in individuals with ongoing viral replication in the absence of antiretroviral combination therapy (Fig. 2 A). In contrast, the proliferative capacity of these cells diminished dramatically during the same time period in antiretroviral therapy–naive individuals, with almost no CD8+ T cells proliferating in response to HIV-1 antigen after 1 yr of follow-up. In individuals with rapid institution of antiretroviral therapy during primary HIV-1 infection (Fig. 2 B), we observed a stable magnitude of interferon γ–secreting, HIV-1–specific CD8+ T cell responses over a 1-yr study period (a median of 1,420 SFCs/10^6 PBMCs vs. a median of 1,985 SFCs/10^6 PBMCs). Interestingly, the corresponding proportions of CD8+ T cells proliferating in response to HIV-1 antigen declined substantially after primary HIV-1 infection, but were maintained at clearly detectable levels and significantly exceeded the proportion of antigen-specific proliferating CD8+ T cells in persons with continuing viral replication (a median of 2.2% [range: 0.7–14.25%] vs. a median of 0.4% [range: 0.3–1.2%], respectively; P = 0.03), although there was no statistically significant difference between these two study cohorts at baseline during primary infection (P = 0.5). In line with previous reports (12, 20), a partial conservation of lymphoproliferative responses by initiation of antiretroviral therapy during primary HIV-1 infection was also observed for HIV-1–specific CD4+ T cells, whereas the HIV-1–specific proliferation of CD4+ T cells was essentially lost 1 yr after acute HIV-1 infection in individuals with continuously ongoing viral replication (Fig. 2, A and B). Overall, we observed a strong correlation between the total proportion of proliferating HIV-1–specific CD4+ and CD8+ cells (Fig. 2 C). In con-

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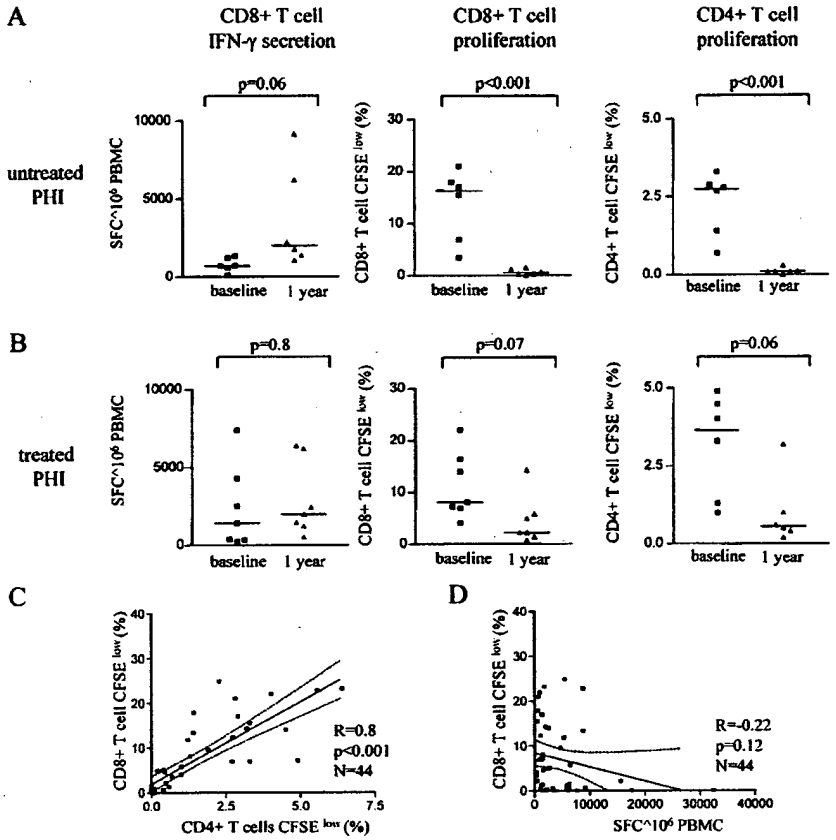

Figure 2. Longitudinal evolution of HIV-1–specific $CD8^+$ T cell proliferative responses after primary HIV-1 infection. (A and B) Simultaneous assessment of antigen-specific proliferation and interferon γ secretion of $CD8^+$ and $CD4^+$ T cells after stimulation with overlapping peptides spanning the entire HIV-1 proteome at baseline and after 1 yr of follow-up in study persons with untreated (A) and treated (B) primary HIV-1 infection. (C and D) Correlation between HIV-1–specific $CD8^+$ and $CD4^+$ T cell lymphoproliferative responses. Proportions of $CD8^+$ cells proliferating after exposure to overlapping peptides spanning the HIV-1 proteome were plotted against the corresponding proportion of $CD4^+$ T cells (C) and against the corresponding magnitude of $CD8^+$ T cell–mediated SFCs/10^6 PBMCs using an interferon γ ELISPOT assay (D). Data from the cross-sectional and longitudinal analysis were included. Dashed lines indicate the 95% confidence interval of the regression line.

trast, no correlation was seen between HIV-1–specific $CD8^+$ T cell lymphoproliferative responses and the corresponding magnitude of HIV-1–specific, interferon γ–secreting $CD8^+$ T cells (Fig. 2 D). Taken together, these data indicate a parallel evolution of lymphoproliferative HIV-1–specific $CD8^+$ and $CD4^+$ T cell responses, and suggest a potential link between the ex vivo proliferative responses of these two antigen-specific T cell populations.

Ex Vivo Proliferation of HIV-1–specific $CD8^+$ Cells Critically Depends on IL-2. Previous studies indicate a decisive role of IL-2 for maintaining the ex vivo proliferative activity of HIV-1–specific $CD4^+$ T helper cells (21). Here, we conducted a series of experiments to elucidate the relevance of IL-2 for also sustaining ex vivo proliferative capacities of HIV-1–specific $CD8^+$ T cells. Overall, the neutralization of IL-2 by IL-2–specific mAbs resulted in an almost complete abrogation of ex vivo proliferative activities of HIV-1–specific $CD8^+$ T cells from study subjects with acute HIV-1 infection, whereas control antibodies did not yield similar effects (Fig. 3, A–D). This was the case both for the entire population of $CD8^+$ T cells dividing after stimulation with HIV-1 peptides (Fig. 3, A and C), as well as for subsets of proliferating $CD8^+$ T cells specific for certain defined HIV-1 $CD8^+$ T cell epitopes, as determined by staining with HIV-1 epitope-specific MHC class I tetramers (Fig. 3, B and D). IL-2–neutralizing mABs similarly abrogated the proliferative capacity of CMV-specific $CD8^+$ T cells (not depicted), indicating that IL-2 dependence was not confined to HIV-1–specific $CD8^+$ T cells. In addition, $CD8^+$ T cells proliferating in response to HIV-1 peptides significantly up-regulated the surface expression of the IL-2Rα chain. In contrast, these dividing $CD8^+$ T cells down-regulated or maintained constant cell surface expression levels of the α receptor chains for the homeostatic cytokines IL-7 and IL-15 (Fig. 3, E and F). Taken together, these results indicate a critical relevance of IL-2 for the ex vivo proliferative capacity of HIV-1–specific $CD8^+$ T cells.

IL-2 Production by $CD4^+$ T Cells Supports HIV-1–specific $CD8^+$ T Cell Proliferation. To more closely determine cell populations supporting the ex vivo proliferative activity of HIV-1–specific $CD8^+$ T cells in individuals with primary HIV-1 infection, we assessed HIV-1–specific $CD8^+$ T cell lymphoproliferative responses after the selective ex vivo removal of distinct leukocyte subsets (Fig. 4, A–C). Interestingly, HIV-1–specific $CD8^+$ T cell proliferation was almost entirely blocked when lymphocyte samples had been depleted of $CD4^+$ cells before the addition of HIV-1 peptides (Fig. 4, A and B). In contrast, HIV-1–specific proliferation was restored after coincubation of isolated $CD8^+$ T cells with isolated $CD4^+$ T cells, indicating that $CD4^+$ T cells are essential for the ex vivo proliferative activities of HIV-1–specific $CD8^+$ T cells (Fig. 4, A and B). The HIV-1–specific proliferative capacity of $CD8^+$ T cells that was lost after depletion of autologous $CD4^+$ cells was also restored by

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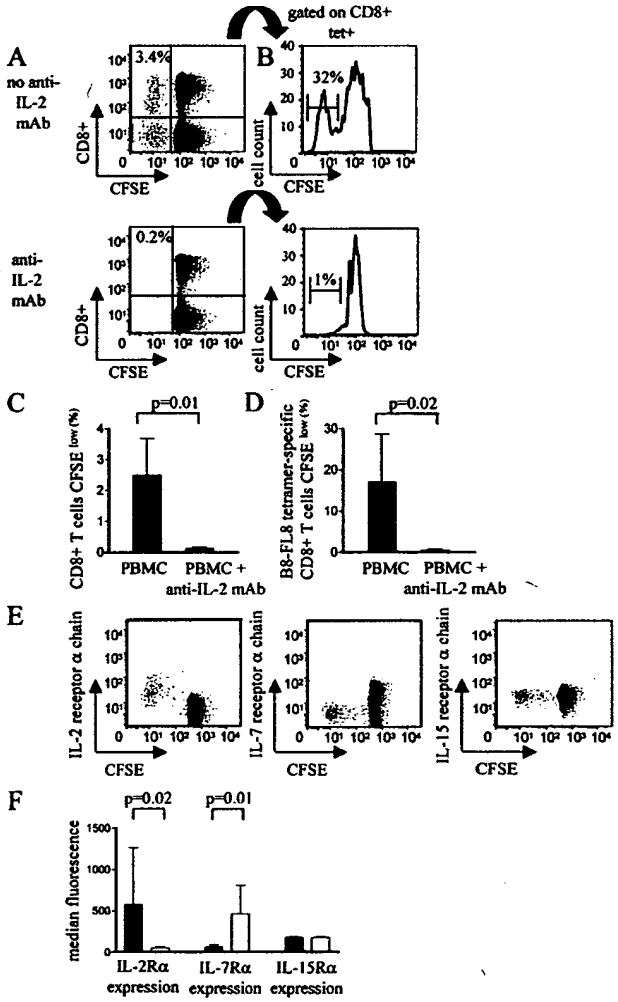

Figure 3. Antigen-specific ex vivo proliferation of HIV-1–specific CD8+ T cells critically depends on IL-2. (A and B) Dot plots (A) or histograms (B) showing the flow cytometric analysis of the proportion of CD8+ T cells (A) or HLA-B8-FLKEKGGL (FL8) tetramer–specific CD8+ T cells (B) proliferating in response to stimulation with a pool of overlapping peptides spanning HIV-1 Nef in the presence or absence of anti–IL-2 mAb. Values in top left corner of dot plots indicate the proportion of $CFSE^{low}$ CD8+ T cells. (C and D) Proportion of CD8+ T cells (C) or B8-FL8 tetramer–specific CD8+ T cells (D) proliferating after exposure to a pool of overlapping Nef peptides in the presence or absence of IL-2 antibodies. Mean and standard deviation of four experiments in four different study persons are shown. (E) Flow cytometric analysis of the surface expression of the α chain of the IL-2, IL-7, and IL-15 receptor in CD8+ T cells proliferating after stimulation with overlapping HIV-1 Nef peptides. (F) Median fluorescence of antibodies directed against the α chain of the IL-2, IL-7, and IL-15 receptor in CD8+ T cells proliferating (black bars) or nonproliferating (white bars) after stimulation with HIV-1 Nef peptides. Data reflect the mean and standard deviation of five independent experiments in four different study subjects.

the addition of exogenous IL-2 (Fig. 4, D–G), indicating that IL-2 can directly support HIV-1–specific CD8+ T cells in the absence of CD4+ T helper cells. In addition, exogenous IL-2 restored the ex vivo proliferative capacity of a subset of HIV-1–specific CD8+ T cells in individuals with chronic progressive HIV-1 infection that was otherwise lost (Fig. 4, H and I). Thus, these data show that the ex vivo proliferative activity of HIV-1–specific CD8+ T cells critically depends on IL-2 secreted by CD4+ T cells.

Antigenic Stimulation of CD4+ T Cells Significantly Enhances the Antigen-specific Proliferative Activity of HIV-1–specific CD8+ T Cells. In our previous experiments, the ex vivo proliferation capacity of HIV-1–specific CD8+ T cells was assessed after stimulation of PBMCs with pools of overlapping peptides spanning HIV-1 proteins, which simultaneously elicited lymphoproliferative CD4+ and CD8+ T cell responses, but did not allow for the analysis of the lymphoproliferative activity of HIV-1–specific CD8+ T cells in the absence of concurrent CD4+ T cell proliferative immune responses. We subsequently tested the ex vivo proliferative capacity of HIV-1–specific CD8+ T cells that had been stimulated with defined optimal HIV-1–specific CD8+ T cell epitopic peptides in the presence or absence of a simultaneous stimulus for HIV-1–specific CD4+ T cell proliferative responses. Fig. 5, A and B, shows results from the HLA-B8–expressing study individual AC-31. Only a limited proportion of CD8+ T cells specific for the HLA-B8–restricted Nef epitope FLKEKGGL (B8-FL8) and virtually no CD4+ T cells proliferated when PBMCs samples were stimulated with the HLA-B8–restricted CD8+ T cell epitopic peptide B8-FL8 alone. In contrast, a dramatically stronger HIV-1–specific CD8+ T cell proliferative response was observed after stimulation of PBMCs with the B8-FL8 peptide and an additional HIV-1 Nef peptide (PEKEVLVWKFDSRLAFHH) that, when used alone, elicited a selective CD4+ T cell–mediated lymphoproliferative response, but no significant CD8+ T cell proliferation (Fig. 5, A, B, D, and E). The enhancement of the ex vivo proliferation of HIV-1–specific CD8+ T cells by synchronized stimulation of HIV-1–specific CD4+ T cells was almost entirely blocked by adding IL-2–neutralizing antibodies (Fig. 5 C). Finally, we observed that the ex vivo proliferation of HIV-1–specific CD8+ T cells can also be enhanced by simultaneous stimulation of CD4+ T cells specific for CMV or tetanus toxoid (Fig. 5, D–F). Thus, these data illustrate that antigen-specific lymphoproliferative CD4+ T cell responses significantly enhance the ex vivo proliferative activity of HIV-1–specific CD8+ T cells in an IL-2–dependent fashion.

Autologous HIV-1–specific CD4+ T Cells Isolated in Acute Infection Can Reconstitute the Proliferative Activity of HIV-1–specific CD8+ T Cells in Chronic Infection. The above data demonstrate that HIV-1–specific CD8+ T cell proliferation critically depends on IL-2 produced by antigen-specific CD4+ T cells. Next, we tested whether CD4+ T cells harvested during acute HIV-1 infection, when triggered by HIV-1, could rescue the ex vivo proliferative activity of HIV-1–specific CD8+ T cells in chronic, untreated HIV-1 infection. Fig. 6 A shows data from study individual AC-98. Strong CD8+ T cell–mediated lymphoproliferative immune responses were observed in acute HIV-1 infection after PBMC stimulation with a pool of overlapping HIV-1 Nef peptides, with a significant proportion of these proliferating

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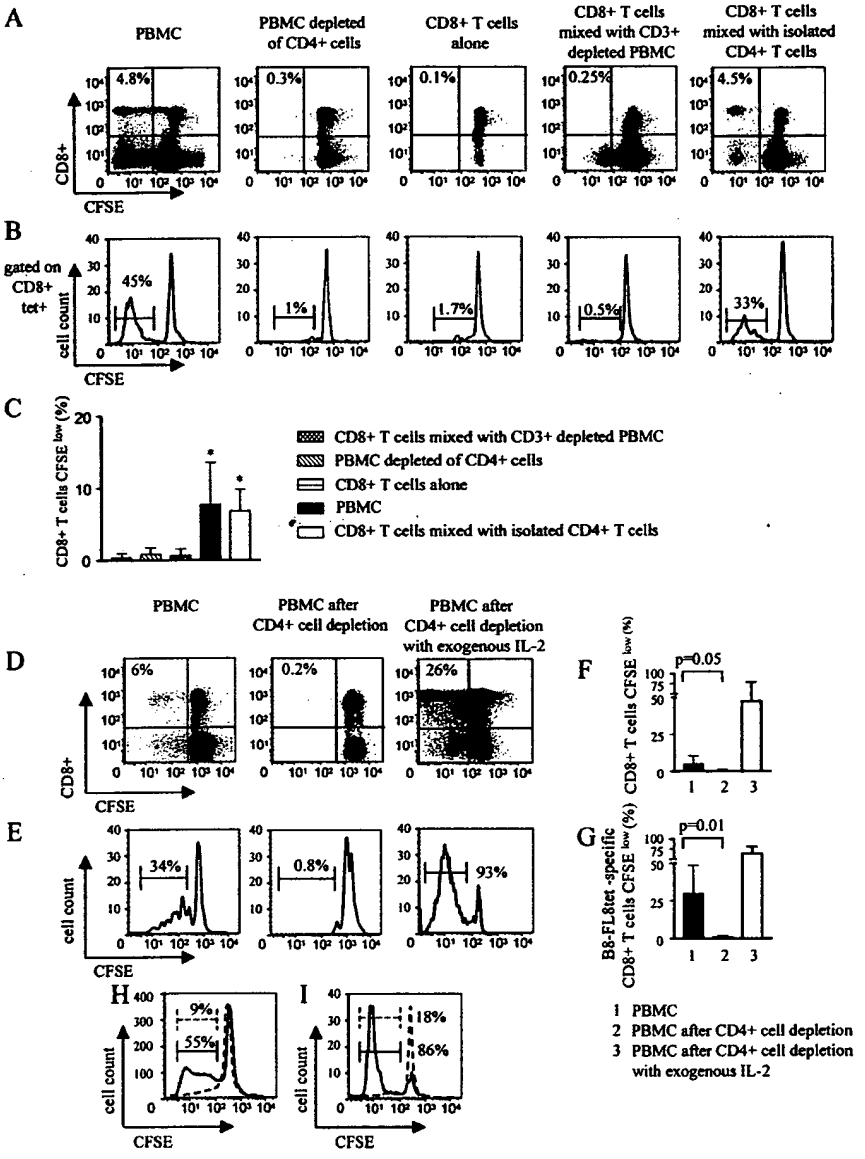

Figure 4. Ex vivo proliferation of HIV-1–specific $CD8^+$ T cells is supported by $CD4^+$ T cells. (A–C) Dot plots (A) and histograms (B) indicating the flow cytometric assessment of $CD8^+$ T cells (A) or B8-FL8–specific $CD8^+$ T cells (B) proliferating after stimulation with a Nef peptide pool in the presence or absence of indicated leukocellular subsets. Cells were gated according to forward scatter (FSC)/side scatter (SSC) characteristics of the lymphocyte population in A. In B, lymphocytes were additionally gated according to $CD8^+$ expression and tetramer binding. Values in top left corner of dot plots indicate the proportion of $CFSE^{low}$ $CD8^+$ T cells. A and B show one representative experiment and C indicates the mean and standard deviation of the proportion of $CFSE^{low}$ $CD8^+$ T cells in four independent experiments (*, $P < 0.05$). (D–G) Dot plots (D) and histograms (E) showing the flow cytometric analysis of the proliferation of $CD8^+$ T cells (D) or B8-FL8–specific $CD8^+$ T cells (E) in responses to stimulation with a Nef peptide pool in whole PBMC samples, $CD4^+$ cell–depleted PBMC samples, and in $CD4^+$ cell–depleted PBMC samples that were supplemented with exogenous IL-2. Gating was performed as described for A and B. D and E show one representative experiment and F and G give the mean and standard deviation from ten independent experiments for bulk $CD8^+$ T cells (F) and three different experiments for tetramer-specific cells (G), respectively. (F and G) Left black bars represent proliferating cells in whole PBMC samples, middle bars show PBMC samples depleted of $CD4^+$ cells, and right white bars indicate PBMC samples depleted of $CD4^+$ cells, but supplemented with exogenous IL-2. (H and I) Rescue of HIV-1–specific $CD8^+$ T cell proliferation by exogenous IL-2 in chronic replicative HIV-1 infection. (H) $CD8^+$ T cell proliferation in the presence of exogenous IL-2 after stimulation of PBMC samples from chronic HIV-1 infection with Nef pool peptides (solid line) or no antigenic stimulation (dashed line). Gating was performed according to FSC/SSC characteristics and $CD8^+$ expression. (I) Proliferation of HLA-A3-QK10 tetramer–specific $CD8^+$ T cells in the presence of IL-2 after stimulation of PBMC samples from chronic HIV-1 infection with Nef pool peptides (solid line) or no antigenic stimulation (dashed line). Cells were gated according to FSC/SSC characteristics, as well as CD8 expression and HLA-A3 QK10 tetramer binding. (H and I) Percentages indicate the proportion of $CFSE^{low}$ $CD8^+$ T cells. One representative example of four different experiments is shown.

cells being specific for the HLA-A3–restricted Nef epitope A3-QK10. These lymphoproliferative responses were almost completely lost during chronic HIV-1 infection, despite the physical preservation of QK10-specific $CD8^+$ T cells, as determined by staining with QK10-specific MHC class I tetramers (Fig. 6, A and B). Yet, the addition of autologous $CD4^+$ T cells isolated during acute HIV-1 infection to the PBMC sample from chronic infection rescued the proliferation of a subset of $CD8^+$ T cells specific for the QK10 tetramer after stimulation with a pool of overlapping Nef peptides, whereas the addition of the same number of autologous $CD4^+$ T cells isolated during chronic HIV-1 infection did not increase the proliferative activity of HIV-1–specific $CD8^+$ T cells in chronic infection. The added autologous $CD4^+$ T cells from acute HIV-1 infection exhibited strong HIV-1 Nef-specific lymphoproliferative capacities and IL-2 secretion (Fig. 6, A and C), whereas $CD4^+$ T cells from the chronic disease phase were neither able to secrete IL-2 nor proliferate in an antigen-specific manner (not depicted). In two additional study subjects, we observed that HLA-A2–restricted $CD8^+$ T cells specific for the p17 Gag epitope SLYNTVATL (SL9) had almost entirely lost their antigen-specific proliferative capacity in chronic infection, despite being detectable at high frequencies, as deter-

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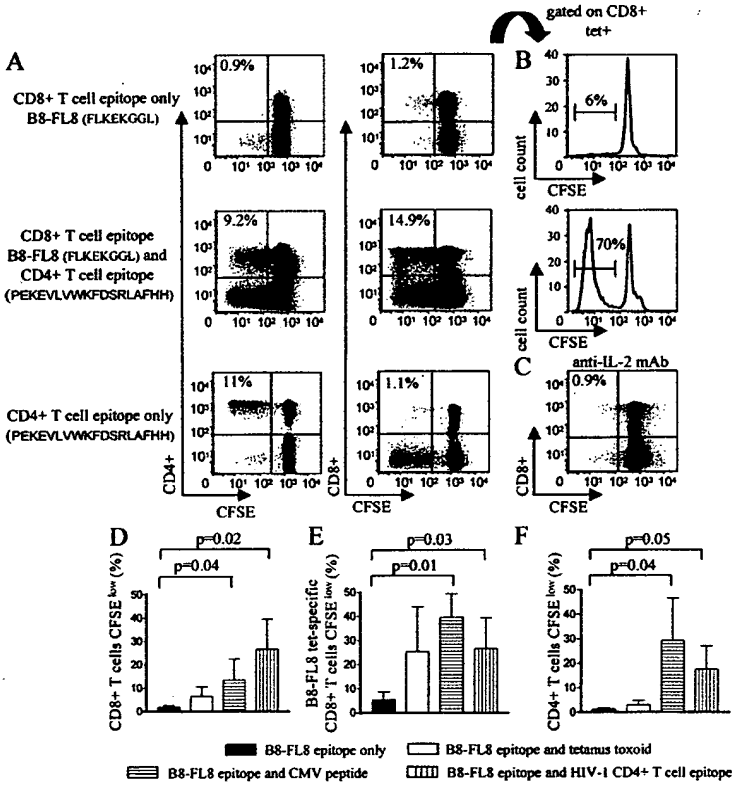

Figure 5. Ex vivo proliferative activities of HIV-1–specific $CD8^+$ T cells are dramatically enhanced by simultaneous stimulation of antigen-specific $CD4^+$ T cells. (A) $CD8^+$ and $CD4^+$ T cell proliferation after stimulation with the HIV-1 Nef $CD8^+$ T cell epitope B8-FL8 or with the overlapping HIV-1 Nef peptide PEKEVLVWKFDSRLAFHH, or both peptides together. Dot plots of one representative flow cytometric experiment are shown. Cells were gated according to FSC/SSC characteristics of the lymphocyte population. (B) B8-FL8 tetramer–specific $CD8^+$ T cells proliferating after stimulation with B8-FL8 peptide only (top) or in conjunction with the overlapping Nef peptide PEKEVLVWKFDSRLAFHH (bottom). (C) $CD8^+$ T cell proliferation after simultaneous stimulation of PBMC samples with the $CD8^+$ T cell epitope B8-FL8 and the HIV-1 Nef peptide PEKEVLVWKFDSRLAFHH in the presence of IL-2 antibodies. Cells were gated according to FSC/SSC characteristics of the lymphocyte population. (D and E) Proportion of $CD8^+$ T cells (D), B8-FL8 tetramer–specific $CD8^+$ T cells (E), or $CD4^+$ T cells (F) proliferating after stimulation with the B8-FL8 epitopic peptide in the presence or absence of concomitant stimulation with tetanus toxoid, a CMV peptide, or the overlapping HIV-1 Nef peptide PEKEVLVWKFDSRLAFHH. Mean and standard deviation from three independent experiments in three different study subjects are shown.

mined by specific staining of $CD8^+$ T cells with SL9–MHC class I tetramer complexes. Yet, after addition of autologous $CD4^+$ T cells harvested during acute HIV-1 infection, the ex vivo proliferative capacities of a subset of these cells were rescued (Fig. 6, D and E). Again, we found that the $CD4^+$ T cells harvested during acute infection included HIV-1–specific $CD4^+$ T cells with strong capacities for ex vivo proliferation and IL-2 secretion after encounter with HIV-1 peptides (not depicted). Moreover, the enhancement of HIV-1–specific $CD8^+$ T cell lymphoproliferative responses by $CD4^+$ T cells isolated during acute HIV-1 infection was almost entirely abrogated by IL-2–neutralizing antibodies (not depicted). Taken together, these results show that $CD4^+$ T cells isolated during acute HIV-1 infection can rescue the ex vivo proliferative capacity of HIV-1–specific $CD8^+$ T cells in chronic HIV-1 infection by an IL-2–dependent mechanism.

In Vivo Reconstitution of the Proliferative Activity of HIV-1–specific $CD8^+$ T Cells by Vaccine-induced, IL-2–secreting, HIV-1–specific $CD4^+$ T Cells. A number of studies have indicated that the administration of inactivated gp120-depleted HIV-1 can result in the induction of strong HIV-1–specific $CD4^+$ T cell lymphoproliferative responses (22–25). We showed recently in a placebo-controlled phase II clinical trial in 10 individuals (5 receiving HIV vaccine and 5 receiving adjuvant alone), that the vaccine could elicit vigorous HIV-1–specific $CD4^+$ T cell–mediated lymphoproliferative immune responses in chronically infected HIV-1 persons treated with highly active antiretroviral therapy, but did not increase the magnitude of HIV-1–specific $CD8^+$ T cell responses when measured by an interferon γ ELISPOT assay (14). We predicted, based on the in vitro data described above, that the in vivo induction of HIV-1–specific $CD4^+$ T cell proliferative responses in these individuals should result in the simultaneous development of HIV-1–specific $CD8^+$ T cell proliferative responses, and used cryopreserved samples from this randomized trial to further characterize the quality of HIV-1–specific $CD4^+$ T cell responses induced by the vaccine, as well as their impact on HIV-1–specific $CD8^+$ T cell responses.

After five consecutive immunizations with gp120-depleted, inactivated HIV, a median of 0.6% (range: 0.4–1.9%) of $CD4^+$ T cells secreted IL-2 in response to stimulation with overlapping peptides spanning HIV-1, whereas IL-2 secretion by $CD4^+$ T cells was minimal in five recipients of placebo (median proportion of 0.1% [range: 0–0.2%]; $P = 0.05$). We subsequently assessed the impact of immunization on HIV-1–specific proliferative T cell responses. In line with our previous data, HIV-1–specific lymphoproliferative activities of $CD4^+$ T cells were negligible at baseline in all 10 individuals with chronic infection. After immunization, HIV-1–specific $CD4^+$ T cells in vaccinees but not in control individuals developed strong proliferative capacities (Fig. 7, A and C), as described previously using a standard tritium-incorporation assay (14). In addition, strong lymphoproliferative activities were also observed in HIV-1–specific $CD8^+$ T cells from vaccine recipients, but virtually no $CD8^+$ T cell–mediated lympho-

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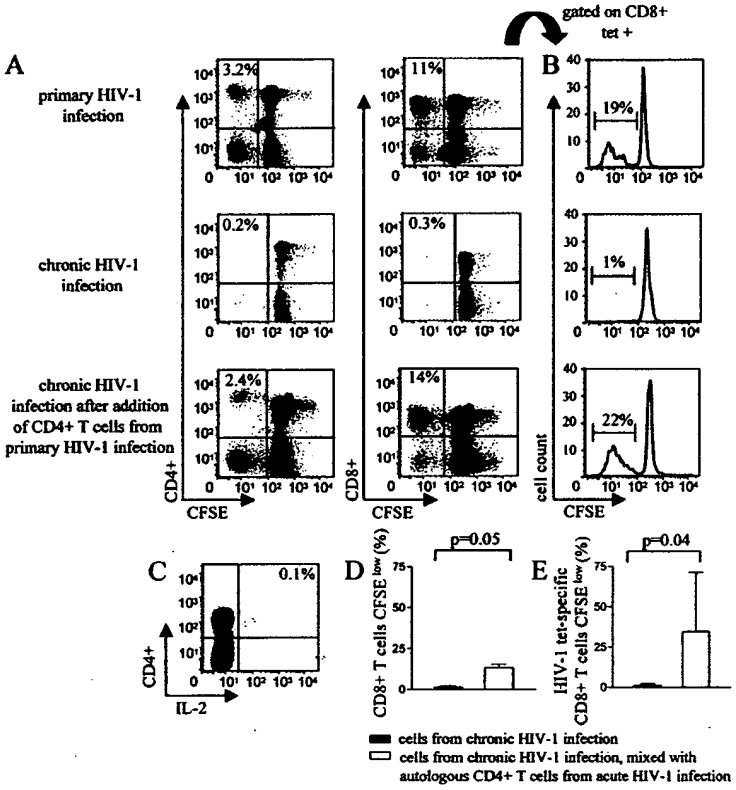

Figure 6. Autologous CD4+ T cells harvested during primary HIV-1 infection can restore the ex vivo proliferative activity of HIV-1–specific CD8+ T cells in chronic HIV-1 infection. (A) Flow cytometric analysis of CD8+ and CD4+ T cell proliferation after stimulation with a pool of overlapping Nef peptides in study subject AC-98 during primary and chronic HIV-1 infection and in chronic HIV-1 infection after the addition of isolated autologous CD4+ T cells harvested during acute HIV-1 infection. Values in top left corner of dot plots indicate the proportion of $CFSE^{low}$ CD8+ or CD4+ T cells, respectively. (B) Corresponding histograms indicating the proportion of proliferating A3-QK10–specific CD8+ T cells. (C) Intracellular cytokine staining of CD4+ T cells from acute HIV infection in study subject AC-98 after stimulation with Nef pool peptides. Cells were gated according to FSC/SSC characteristics. Value in top right corner indicates proportion of IL-2+ CD4+ T cells. (D and E) Proportion of CD8+ T cells (D) and HIV-1 tetramer–specific CD8+ T cells (E) proliferating in chronic HIV-1 infection after stimulation with a pool of HIV-1–specific Nef peptides in the presence (white bars) or absence (black bars) of added autologous CD4+ T cells from acute HIV-1 infection. Data indicate the mean and standard deviation from three study individuals described in Results.

proliferative activities were observed in control individuals (Fig. 7, B and D). CD4+ and CD8+ T cell–mediated lymphoproliferative responses in vaccinees were confined to those antigens contained in the immunogen, with no induction of responses to envelope proteins that were removed during the preparation of the antigen (26). Thus, these data indicate that the in vivo augmentation of virus-specific CD4+ T cell responses can lead to the reconstitu-

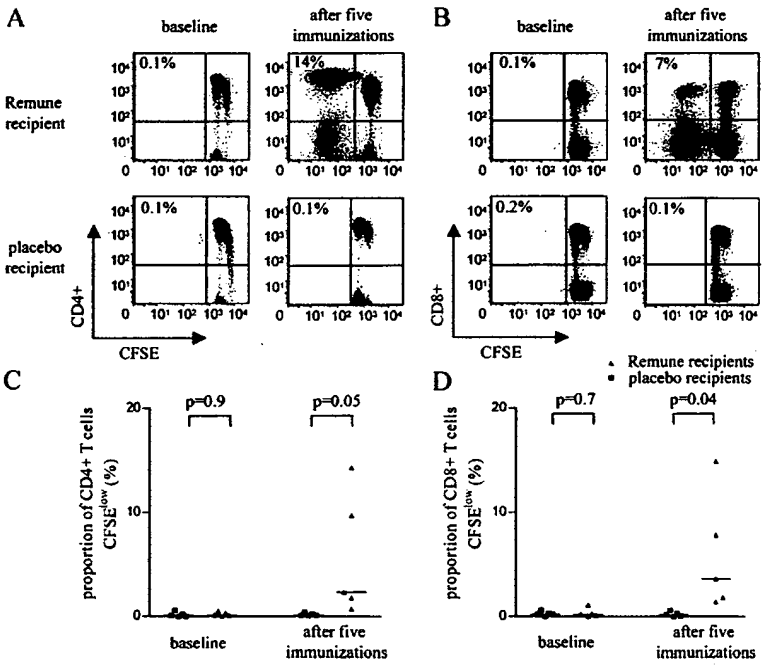

Figure 7. In vivo reconstitution of HIV-1–specific CD8+ T cell lymphoproliferative activities by vaccine-mediated induction of IL-2–secreting, HIV-1–specific CD4+ T cells. (A and B) Dot plots reflecting the lymphoproliferative activity of HIV-1–specific CD4+ (A) and CD8+ T (B) cells after stimulation with a pool of overlapping Gag peptides before and after five consecutive administrations of an Env-depleted immunogen or placebo. Percentages indicate the proportion of $CFSE^{low}$ CD8+ and CD4+ T cells. (C and D) Proportions of CD4+ (C) and CD8+ (D) T cells proliferating in response to stimulation with HIV-1 peptides spanning the entire HIV-1 proteome in five recipients of placebo and the vaccine. Data from baseline and after five consecutive administrations of the immunogen/placebo are shown.

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tion of HIV-1–specific $CD8^+$ T cell lymphoproliferative immune responses in vivo.

Discussion

HIV-1–specific $CD8^+$ T cells play a critical role in the initial control of viral replication in acute infection (27, 28). Yet, the functional correlates for $CD8^+$ T cell–mediated HIV-1 immune control are not well understood. Here, we show that HIV-1–specific $CD8^+$ T cells in acute HIV-1 infection exhibit strong ex vivo proliferative capacities, whereas this effector function is rapidly lost in the presence of ongoing viral replication. Moreover, our data demonstrate that lymphoproliferative $CD4^+$ T cell responses enhanced HIV-1–specific $CD8^+$ T cell proliferation in an IL-2–dependent fashion, whereas no HIV-1–specific $CD8^+$ T cell proliferation was observed in individuals with acute infection after in vitro depletion of $CD4^+$ T cells. Finally, the proliferative defect of HIV-1–specific $CD8^+$ T cell responses in chronic infection was partially corrected in vitro by adding autologous IL-2–secreting $CD4^+$ T cells isolated during acute infection and in vivo by the induction of HIV-1–specific $CD4^+$ T cells using an Env-depleted immunogen. Thus, these data demonstrate a progressive loss of HIV-1–specific $CD8^+$ T cell function that is closely linked to the loss of HIV-1–specific, IL-2–secreting $CD4^+$ T cells, but can be rescued in vitro and more importantly in vivo by reconstituting HIV-1–specific $CD4^+$ T cell help.

Recent data have demonstrated that HIV-1–specific $CD8^+$ T cell responses measured by their ability of antigen-specific interferon γ secretion do not differ in individuals with progressive and long-term nonprogressive HIV-1 infection and are not directly associated with the level of viral replication (7, 9). In contrast, HIV-1–specific $CD8^+$ T cells in individuals with long-term nonprogressive infection exhibit strong antigen-dependent ex vivo proliferative capacities, whereas HIV-1–specific $CD8^+$ T cells in subjects with progressive disease courses lose their abilities to proliferate ex vivo in an antigen-specific manner (10). Here, we extend these findings, demonstrating that strong HIV-1–specific $CD8^+$ T cell–mediated lymphoproliferative immune responses are present in acute HIV-1 infection, when high level plasma viremia declines after the first appearance of cellular immune responses against HIV-1. HIV-1–specific $CD8^+$ T cell proliferation was rapidly lost in individuals with ongoing viral replication, despite the persistence of or an increase in the number of interferon γ–secreting, HIV-1–specific $CD8^+$ T cells. In contrast, early suppression of viral replication by antiretroviral therapy preserved these proliferative responses. These data demonstrate that the ability of HIV-1–specific $CD8^+$ T cells to proliferate in response to antigenic stimulation ex vivo can be conserved in individuals with suppressed HIV-1 viremia, but is lost rapidly after acute infection in the presence of ongoing viral replication.

It has recently been shown that the lack of proliferative capacity of HIV-1–specific $CD4^+$ T cells in chronic HIV-1 infection is associated with diminished IL-2 secretion by these cells (13, 21), suggesting a potential relevance of autocrine IL-2 secretion for maintaining HIV-1–specific $CD4^+$ T cell lymphoproliferative responses. In addition, antigen-dependent IL-2 secretion of HIV-1–specific $CD4^+$ T cells is present in acute HIV-1 infection, but sequentially lost during the ensuing disease process, which is closely paralleled by the loss of $CD4^+$ T cell lymphoproliferative immune responses (13, 20). Here, we show that the loss of the proliferative capacity of HIV-1–specific $CD4^+$ T cells is similarly paralleled by a loss of lymphoproliferative HIV-1–specific $CD8^+$ T cell responses, suggesting a mutual functional interaction of these T cell subsets. Moreover, our in vitro data demonstrate that HIV-1–specific $CD4^+$ T cells isolated from acute HIV-1 infection partially restored lymphoproliferative capacities of HIV-1–specific $CD8^+$ T cells in chronic HIV-1 infection by an IL-2–dependent mechanism, whereas no $CD8^+$ T cell proliferation was seen in the presence of $CD4^+$ T cells from chronic HIV-1 infection, which had lost both their antigen-specific lymphoproliferative activity and their ability to secrete IL-2. More importantly, therapeutic immunization aimed at induction of HIV-1–specific $CD4^+$ T cells was able to repair the proliferation deficiency of HIV-1–specific $CD8^+$ cells in vivo in persons with chronic infection. In fact, the administration of a $CD4^+$ T cell–targeted vaccine resulted in HIV-1–specific $CD8^+$ T cell responses with similar lymphoproliferative capacities, as in individuals with acute or long-term nonprogressive HIV-1 infection. Thus, our data suggest that the minimal proliferative capacities of HIV-1–specific $CD8^+$ T cells in chronic HIV-1 infection are not primarily due to a functional defect of these cells, but are rather related to insufficient support by HIV-1–specific $CD4^+$ T helper cells. Nevertheless, even after providing IL-2–secreting $CD4^+$ T helper cells, not all HIV-1–specific $CD8^+$ T cells proliferated, suggesting that within the entire HIV-1–specific $CD8^+$ T cell compartment, populations with different thresholds for antigen-specific proliferation exist.

Our data identify IL-2 secretion as the most prominent mechanism used by $CD4^+$ T helper cells to support HIV-1–specific $CD8^+$ T cell lymohproliferative responses, as IL-2–neutralizing antibodies fully abrogated the proliferative enhancement mediated by $CD4^+$ T cells. This observation is in line with previous data showing that exogenous IL-2 can correct cell cycle perturbations, normalize the overall intracellular protein turnover, and restore the phase-specific pattern of the expression of cell cycle–dependent proteins of lymphocytes in HIV-1–infected individuals (29, 30). However, although IL-2 administration together with highly active antiretroviral therapy has been shown to result in a significant increase in $CD4^+$ T cell counts, it was not associated with enhancement of HIV-1–specific T cell responses or immune-mediated control of HIV-1 infection (31, 32). These data suggest that the HIV-1–specific immune response depends less on systemic levels of IL-2, but rather on the levels of IL-2 provided in the microenvironment of the antigen-specific interaction between antigen-

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presenting cells, virus-specific $CD4^+$ T cells, and virus-specific $CD8^+$ T cells. This is further supported by our in vivo data demonstrating increased HIV-1–specific $CD8^+$ T cell proliferation after the induction of IL-2–secreting, HIV-1–specific $CD4^+$ T cell responses, strengthening the conclusion that IL-2 secretion in an antigen-specific manner appears to be more relevant for the maintenance of HIV-1–specific proliferative $CD8^+$ T cell responses than systemic levels of IL-2.

Taken together, these data demonstrate a parallel impairment of both HIV-1–specific $CD4^+$ and $CD8^+$ T cell proliferative responses after acute HIV-1 infection in the presence of ongoing viral replication, and suggest a critical role of IL-2–secreting $CD4^+$ T helper cells for sustaining the proliferative capacity of these HIV-1–specific T cell responses both in vitro and in vivo. These results provide evidence for direct functional linkage of HIV-1–specific $CD4^+$ and $CD8^+$ T cell responses and contribute to the understanding of key molecular events contributing to the immunopathogenesis of HIV-1 infection.

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Therapeutic immunization with an inactivated HIV-1 Immunogen plus antiretrovirals versus antiretroviral therapy alone in asymptomatic HIV-infected subjects

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Abstract

To determine whether the addition of an inactivated-gp120-depleted HIV-1 Immunogen to antiretrovirals (ARTs) conferred a beneficial effect on delaying time to virologic failure relative to that obtained by ARTs alone, a phase II clinical trial was performed in 243 asymptomatic, ART naïve, HIV-1 seropositive adults. The Cox model showed that HIV-1 Immunogen treatment was associated with a 34% decrease in the risk of virologic failure ($P = 0.056$). When the analysis incorporated baseline HIV-RNA stratification the risk of virologic failure in the HIV-1 Immunogen Arm was significantly reduced a 37% compared to the IFA placebo Arm ($P = 0.034$). The data suggest that therapeutic immunization plus ARTs could influence virologic control.
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Keywords: HIV-1 Immunogen; Viral load; Therapeutic vaccination

1. Introduction

Antiretroviral (ART) therapies have significantly lowered morbidity and mortality. However, prolonged antiretroviral therapy does not appear to eradicate viral reservoirs [1] and is not sufficient to restore completely immune competence [2]. In addition, the emergence of resistance as well as their long-term toxicities have warranted new approaches, such as therapeutic immunization [3,4].

Previous studies using various therapeutic regimens involving the HIV-1 Immunogen (REMUNE™) in combination with antiretrovirals have shown the ability to stimulate HIV-specific lymphoproliferative responses (LPR), with

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EXHIBIT D

no serious side effects, in patients with HIV-1 chronic infection [5–8]. A previous clinical trial using the HIV-1 Immunogen in combination with ARTs in a population of adults with chronic HIV-1 infection did not provide evidence of a clinical protective effect (progression to AIDS or death) [9]. We have examined throughout a period of 3 years whether administration of HIV-1 Immunogen plus a standard fixed antiviral drug combination impacted on delaying viral rebound or CD4+ T cell decline in a homogeneous population of ART naïve, asymptomatic patients.

It was hypothesized that it might be possible by immunizing with an HIV-1 Immunogen to reconstitute HIV-specific memory cell-mediated immunity in patients with chronic HIV-1 infection that could improve the rate of sustained virologic suppression in patients on ARTs. This study was also designed to determine whether therapeutic vaccination with the HIV-1 Immunogen can reconstitute HIV-1-specific T helper lymphocyte proliferative responses. In addition to previous studies, we have included mitogens and alloantigens to assess whether HIV-1-antigen immune responses were specific. Moreover, we have analyzed the correlation of the induction of HIV-1-specific LPR with Th1 cytokine and beta-chemokine production.

2. Materials and methods

2.1. Study design and population

Study STIR-2102 was a multicenter, randomized, double-blind, placebo-controlled phase II clinical trial of HIV-1 Immunogen plus ARTs versus ARTs alone in HIV-1 infected subjects. Asymptomatic, ART naïve, HIV-1 seropositive individuals ($n = 243$) were enrolled into this study at 13 clinical centers across Spain. Institutional Review Board approval and informed consents from all participants were obtained prior to study commencement. All the subjects recruited in this trial were required to meet the following criteria: male or non-pregnant female of 18 years or older; serodiagnosis with HIV infection documented by a positive ELISA antibody test and confirmed by Western blot; CD4 T cell counts between 300 and $700 \times 10^6\,l^{-1}$ from two CD4 cell assessments taken 4 weeks apart and performed within 30 days prior to study randomization; ARTs naïve; asymptomatic (exclusion of any HIV-related conditions, with the exception of oral candidiasis and oral hairy leukoplakia), and other standard laboratory and clinical inclusion and exclusion criteria (not shown). In addition, prior to randomization, study subjects that did not show a decrease in HIV-RNA of less than 5000 copies per ml from baseline, after 6 weeks of pre-treatment with ARTs, were not randomized. Four out of the total number of enrolled patients (243) were not eligible, because they did not comply with this last requirement.

2.2. Interventions

The study drug was the HIV-1 Immunogen that consisted of gp120-depleted, inactivated HIV-1 at a dose of 10 units of p24 antigen in incomplete Freund's adjuvant (IFA). HIV-1 Immunogen was obtained from a Zairian HIV isolate (HZ321), clade A envelope and clade G gag, grown in a lymphoblastoid cell line (HUT-78-cell line) and purified as has been previously described [10–12]. For in vitro experiments, native p24 was preferentially lysed from purified and inactivated HIV-1 with 2% Triton X-100 being purified afterwards by using Pharmacia Sepharose fast flow S resin. The placebo consisted of incomplete Freund's adjuvant. Both the HIV-1 Immunogen and the IFA were manufactured and supplied by the study sponsor (Immune Response Corporation [IRC], Carlsbad, CA) in single use pre-filled syringes. The clinical trial was initiated on January 1997. Patients started therapy 6 weeks prior to randomization on a standard fixed two antiviral drug combination (ART) of two nucleoside reverse transcriptase inhibitors (NRTIs), including zidovudine (AZT) and didanosine (DDI). After the introduction of protease inhibitors (PIs), the protocol design was amended to allow subjects to receive the fixed three antiviral drug combination (HAART) of two NRTIs, lamivudine (3TC) and stavudine (D4T), and one PI, Indinavir (IDV). At the time of randomization (day 0), 100% of the patients were receiving ART (AZT/DDI). The total population that switched to HAART (3TC/D4T/Indinavir) throughout the study was 123 (52%) subjects, 60 (50.9%) subjects in HIV-1 Immunogen Arm and 63 (52.1%) subjects in the IFA placebo Arm (Fisher's exact test, $P > 0.99$). The reasons to switch to HAART were toxicity or intolerance to the combination ART, mainly DDI, pregnancy or persistent high viral load without reaching an endpoint. This later criteria occurred only in 7/60 (11.7%) subjects of HIV-1 Immunogen Arm and 8/63 (12.7%) subjects of the IFA placebo Arm (Fisher's exact test, $P > 0.99$).

2.3. Enrollment and study measurements

Patients were randomized in equal proportions to either the HIV-1 Immunogen or IFA Arm using permuted blocks, prior to the administration of the first injection of the HIV-1 Immunogen (day 0). The randomization scheme was generated and maintained by Nufarm 21. The protocol included the stratification of the randomized subjects by baseline CD4 cell count ($<350 \times 10^6$ cells per liter versus >350 to $<450 \times 10^6$ cells per liter versus $>450 \times 10^6$ cells per liter) and baseline viral load (<10,000 copies per milliliter versus >10,000 copies per milliliter). Concerning the masking of the drug, the syringes containing REMUNE™ or IFA had an equal and anonymous appearance and were labelled identically and distributed by an independent third party. Randomisation was considered day 0 of the study. At day 0 and thereafter every 12 weeks, patients treated

with ART or HAART received immunizations with intramuscular injections of the HIV-Immunogen or the adjuvant placebo (IFA), throughout a period of 36 months. Laboratory tests, including CD4 cell counts, plasma HIV-1 RNA levels and immunologic and virologic analyses were also carried out every 3 months. Plasma levels of HIV-1 RNA were assessed using the Amplicor assay with a lower limit of quantification of 200 copies per milliliter (Hoffman La Roche, Nutley, NJ). CD4+ T cell count in peripheral blood was determined by three-colour multi-parameter flow cytometry in a FACScalibur (Becton & Dickinson) cytometer.

2.4. Immunological substudy

An immunologic substudy was conducted within study STIR-2102 in a subset of 60 patients (30 were in the HIV-1 Immunogen Arm and 30 were in the control Arm). The subset of patients were randomly selected at four sites, and analysis were carried out every 6 months (from week 6 to month 36) to determine whether the immunization with the HIV-1 Immunogen could reconstitute HIV-1-specific T helper LPR. Cellular proliferation assays were performed culturing the patient's peripheral blood mononuclear cells (PBMCs) with HIV-1 antigens for 6 days and was evaluated by incorporation of [^{3}H] thymidine to DNA. PBMCs were seeded for triplicate in flat-bottom (culture with mitogens) or U-bottom (culture with antigens) 96-wells plates with RPMI-1640 containing 10% human AB serum. The results were expressed as "lymphocyte stimulation index" (SI), which is the geometric mean counts per minute (cpm) of the cells incubated with antigen divided by the geometric mean cpm of the cells with media alone [6].

HIV-1 antigens used for the lymphocyte proliferation assays included: HIV-1 HZ321 antigen (HIV-1 Immunogen; IRC); native p24 protein fractionated from HZ321 (IRC) and HIV-1 BaL antigen (Clade B env, Advanced Biotechnologies Inc., Columbia, MD, USA) and a mixture of three synthetic *env* peptides of HIV-1 (T1, $P18_{IIIB}$ and $P18_{MN}$, 10 μM). Non-Immunogenic peptide p23 as negative control of *env* peptides was also included. Non-specific lymphocyte proliferation was studied with phytohemagglutinin (PHA, 1 μg/ml: Murex Biotech Limited, Dartford, England) and pokeweed mitogens (PWM, 4 μg/ml: Sigma, St. Louis, MO, USA), and anti-CD3 monoclonal antibody (10 μg/ml, SPV3Tb, kindly provided by Dr. JE de Vries, DNAX, Palo Alto, CA, USA). Alloreactivity was measured in a mixed lymphocyte culture (MLC of a mixture of mitomicin-treated PBMCs of six unrelated donors). In addition, beta-chemokine [macrophage inflammatory protein (MIP-1beta) and RANTES (regulated on activation, normal T-cell expressed and secreted): Endogen, Cambridge, USA] and cytokine production [interferon (IFN)-gamma: CLB, Amsterdam, The Netherlands] in supernatants of HZ321 HIV-1-antigen-stimulated PBMCs cultures were measured by enzyme linked immunoadsorbent assay.

2.5. Efficacy end points and statistical analysis

The primary efficacy end point was defined to be time to virologic and/or immunologic failure: for subjects on the two drug combination of AZT/DDI, was time to the first increase of viral load above 5000 copies per milliliter; for subjects with three drug combination of 3TC/D4T/Indinavir was time to increase of viral load above 2000 copies per milliliter; or a decrease of CD4+ count below 250 cells/mm^3 or a decrease of 50% below baseline. The end point had to be confirmed by a test done 4 weeks after the trigger test. The Wilcoxon test and Fisher exact test were used to compare treatment arms with respect to baseline characteristics. Primary endpoint analysis utilized an intent to treat approach measuring time to failure of subjects. The statistical analysis plan utilized the Kaplan–Meier survival estimates by treatment and the Cox proportional hazards model. Subjects who had not yet developed a primary end point or who were lost to follow-up by the end of the trial (29 May 2001) were censored at the last available visit. Association between variables was measured by means of the Pearson correlation test.

2.6. Roles of the sponsor, investigators and clinical research organization

A clinical protocol was established in 1995 by the study chair (E.F.-C.) and the late Dr. Jonas Salk. The study team to independently conduct and analyze the study consisted of the study chair (E.F.-C.), the study co-chairs (J.G.-L. and S.M.) and the study statistician (F.L.). The study sponsor (IRC) contracted with MDS Pharma, former Phoenix International (Madrid, Spain), a clinical research organization (CRO) responsible for the medical monitoring of sites. Nufarm 21 (Madrid, Spain) was the CRO responsible for statistical analysis and for data management. The IRC provided all financial support for this study, Immunogen and IFA. The study team generated, maintained and analyzed all the data presented in this manuscript.

2.7. Interim monitoring

An initial DSMB meeting was held in January 1997. Four interim efficacy/safety analyses were prepared for presentation to the DSMB in 12 November 1998; 15 October 1999; 16 November 2000 and 29 May 2001 in which the DSMB recommended that the trial should be unblinded based on the information provided by the available data. The DSMB requested additional analysis on the same DSMB's database (29 May 2001) that was presented at the last DSMB meeting on 27 July 2001, previously analysed by three independent statisticians.

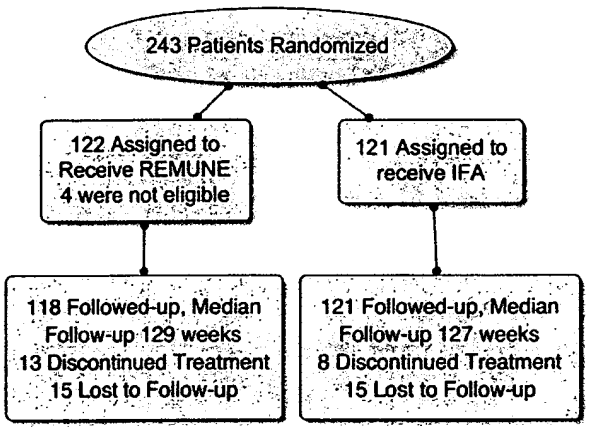

Fig. 1. Profile of the randomized clinical trial. IFA: incomplete Freund's adjuvant.

3. Results

3.1. Randomization of subjects

HIV-1 seropositive patients (n = 243) were enrolled into this study from a total of 435 subjects screened between January 1997 and April 1998. Fig. 1 shows the profile of the randomized clinical trial. A total of 118 (97%) and 121 (100%) patients were followed-up in the study Arm of Immunogen and IFA (placebo), respectively. Lost to follow-up was 15 (13%) and 15 (12%) patients; and 13 (11%) and 8 (7%) patients discontinued treatment in the Immunogen Arm and IFA placebo Arm, respectively. The median follow-up time was 129 weeks. The baseline characteristics including demographic and stratification factors are shown in Table 1. Stratification factors corresponded to baseline (pre-ART and pre-study drug administration) measurements; treatment arms showed no differences in baseline factors: viral load (t-test, P = 0.88), CD4 absolute counts (P = 0.70) and CD4 % (P = 0.42). The distribution of HIV-related diseases was four oral candidiasis (3.5%) in HIV-1 Immunogen Arm, and five oral candidiasis, one tuberculosis and one lymphoma (5.8%) in IFA placebo Arm.

3.2. Evaluation of primary endpoint

A total of 85 participants experienced the primary end point in this trial, of which 84 were due to virologic failure. The rate of endpoints in the HIV-Immunogen and IFA arms were 30 and 41%, respectively. The Kaplan–Meier survival analysis (Fig. 2) showed that there was a trend suggesting a delay in the time to reach virologic failure in the Immunogen Arm versus IFA placebo Arm (Log-rank test: P = 0.056). The data suggests a delay of at least 1 year in virologic rebound in the HIV-1 Immunogen Arm compared to the placebo. The 25th percentile time to the primary endpoints was reached at 367 days in the IFA Arm compared to 753 days in the HIV-Immunogen

Table 1
Baseline characteristics for STIR-2102

	HIV-1 Immunogen	IFA placebo	Overall
No. of subjects	118	121	239
Demographic variables			
Age, mean (S.D.) (years)	33 (7)	35 (8)	34 (7)
Weight, mean (S.D.) (kg)	69.5 (12.9)	71.0 (15.6)	70.3 (14.3)
Gender, N (%)			
Male	85 (72.0)	86 (71.1)	171 (71.5)
Female	33 (28.0)	35 (28.9)	68 (28.5)
Risk group, N (%)			
Homosexual/bisexual	22 (18.6)	28 (23.1)	50 (20.9)
IV drug user	63 (53.4)	47 (38.8)	110 (46.0)
Heterosexual w/HIV+	26 (22.0)	41 (33.9)	67 (28.0)
Unknown	2 (1.7)	1 (0.8)	3 (1.3)
Multiple risks	5 (4.2)	4 (3.3)	9 (3.8)
Strata covariates (baseline, pre-ART values)			
CD4 cells, mean (S.D.), cells $\times 10^6 l^{-1}$	404 (72)	408 (77)	406 (74)
CD4, mean (S.D.) (%)	24 (6)	25 (7)	25 (7)
CD4 strata, N (%)			
<350	31 (26.3)	30 (24.8)	61 (25.5)
350–449	57 (48.3)	59 (48.8)	116 (48.5)
≥450	30 (25.4)	32 (26.4)	62 (25.9)
HIV-RNA, mean (S.D.), $\log_{10}$ copies per ml	4.0 (0.8)	4.0 (0.8)	4.0 (0.8)
HIV-RNA strata, N (%)			
<10,000	55 (46.6)	59 (48.8)	114 (47.7)
≥10,000	63 (53.4)	62 (51.2)	125 (52.3)

IFA: incomplete Freund's adjuvant; HIV: human immunodeficiency virus; S.D.: standard deviation.

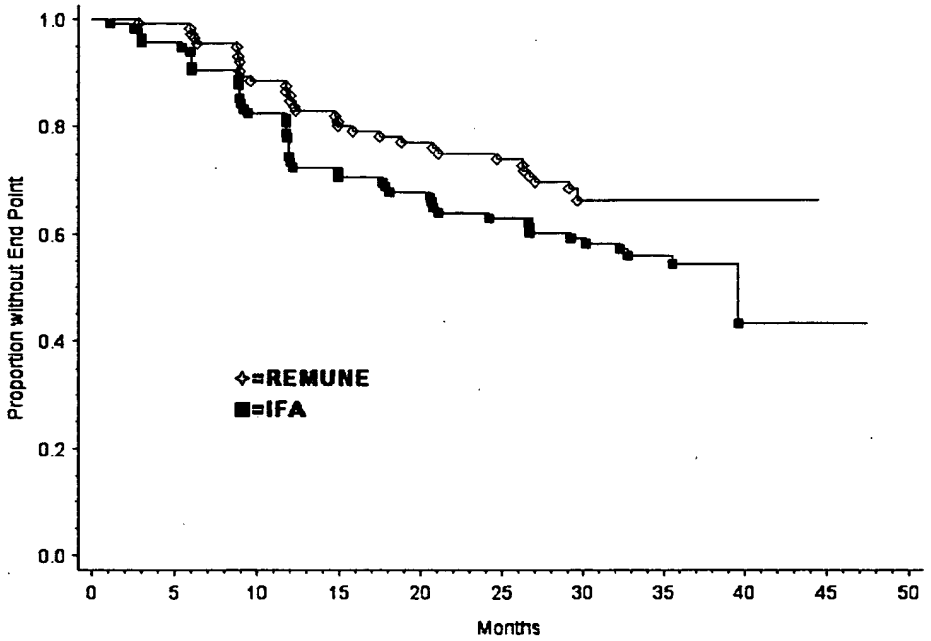

Fig. 2. Kaplan–Meier plot of Immunogen (hollow diamond) vs. placebo (solid square). Estimation of percent of patients without endpoint (y-axis) per study month (x-axis).

Arm. The median time for reaching an endpoint in the IFA Arm was 1205 days and could not be estimated in the HIV-Immunogen Arm because if the difference between the two arms would have been maintained, the estimated median time to virologic failure in the HIV-Immunogen Arm would have greatly exceeded the observation time of the study.

The Cox unadjusted model showed that HIV-1 Immunogen treatment was associated with a 34% decrease in the risk of virologic failure (Table 2). Regression analyses adjusting for baseline HIV-RNA stratification showed that the risk of virologic failure in the HIV-1 Immunogen Arm was significantly reduced (37%) compared to the IFA placebo Arm. When the analysis included both baseline CD4 and HIV RNA stratification factors, the overall virologic failures were significantly reduced (38%) in comparison to the IFA placebo Arm (Table 2).

Table 2
Hazard ratio from Cox proportional regression analysis of virological failure associated with factors including study treatment, CD4 count and HIV-RNA strata

Factors studied	RH (95% CI)	P-value
HIV-1 Immunogen/IFA	0.66 (0.43-1.01)	0.056
HIV-1 Immunogen/IFA + VL strata	0.63 (0.41-0.97)	0.034
HIV-1 Immunogen/IFA + CD4 + VL strata	0.62 (0.40-0.95)	0.029

IFA: incomplete Freund's adjuvant; RH: relative hazard; CI: confidence interval.

3.3. Immunological results substudy

Within 4 weeks of the initial immunization, statistically significant differences were observed between the two groups when they were compared for the level of LPR to HIV-1 antigens, p24, as well as BaL HIV-1 antigens, reaching a plateau at month 18th. Significant differences between both groups were detected along the 36-month follow-up study (Fig. 3). Average SI and standard deviations for the two groups at month 36th are shown in Table 3, demonstrating consistent LPR augmentation in HIV-1 Immuno-

Table 3
Cellular proliferation assays in patients treated with ART alone (IFA group) vs. ART + REMUNE™

	IFA			REMUNE™			P-value
	n	Mean	S.D.	n	Mean	S.D.	
HIV-1 antigens	30	4.9	1.4	30	26.1	5.1	<0.05
p24 antigens	30	2.9	3.9	30	13.5	3.9	<0.05
Bal antigens	30	1.6	0.6	30	6.8	1.7	<0.05
env HIV-1 peptides	30	1.16	0.15	30	1.44	0.25	0.34
PHA mitogen	30	122.4	17.4	30	111.6	14.6	0.56
PWM mitogen	30	72	12.6	30	51.2	9.2	0.16
Anti-CD3	30	84.4	15.3	30	80.1	15.9	0.85
MLC	30	51.7	19.1	30	48.3	21.6	0.9

Mean lymphocyte stimulation index at month 36th. Spanish Phase II study. IFA: incomplete Freund adjuvant; n: number of patients; S.D.: standard deviation; HIV-1 antigens: gp120-depleted HIV-1 (HZ321), clade A envelope and clade G gag; Bal antigen: clade B env; env HIV-1 antigens: T1, $P18_{IIIB}$ and $P18_{MN}$; PHA: phytohemagglutinin; PWM: pokeweed; MLC: mixed lymphocyte culture.

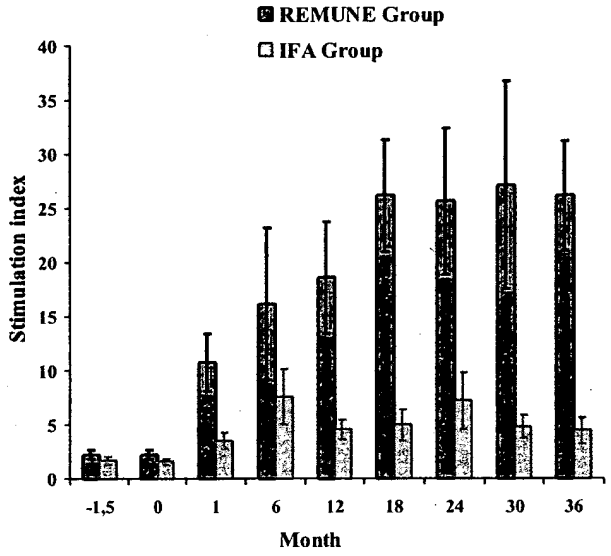

Fig. 3. Cellular proliferation assays in patients treated with ART + REMUNE™ vs. ART alone (IFA group). The HIV-1 Immunogen enhanced significantly HIV-1-specific T cell responses. LPR are expressed as lymphocyte stimulation index. HIV-1 antigens: gp120-depleted HIV-1 (HZ321), clade A envelope and clade G gag.

gen recipients compared to IFA group. Moreover, LPR to HIV-1 antigens correlated positively with LPR to np24 ($R = +0.80$, $P < 0.0005$) and with LPR to BaL HIV-1 antigens ($R = 0.81$, $P < 0.0005$). In contrast, there was no difference between HIV-1 Immunogen recipients and IFA group in the magnitude of SI when PBMCs were stimulated with *env* HIV-1 peptides (T1, $P18_{IIIB}$ and $P18_{MN}$), mitogens PHA and PWM, anti-CD3 monoclonal antibody or in MLC (Table 3). Significant increases in the production of IFN-gamma, MIP-1beta and of RANTES were observed only in the HIV-1 Immunogen Arm as shown in Fig. 4. LPR to HIV-1 antigens correlated positively with production of IFN-gamma ($R = +0.87$; $P < 0.05$), MIP-1beta ($R = +0.72$; $P < 0.05$) and RANTES ($R = +0.72$; $P < 0.05$) in the HIV-1 Immunogen Arm but not in the controls.

3.4. Safety and tolerability

HIV-Immunogen was well tolerated and safe in this trial. A total of 56 patients experienced one or more serious adverse events in the study, of which 27 (23%) were in the IFA-control Arm and 29 (25%) were in the HIV-1 Immunogen Arm ($P = 0.76$). Relationship with the study drug was deemed none by investigators in all cases.

4. Discussion

The results of STIR-2102 trial demonstrates that the appearance of virologic failure among the Arm of patients assigned to the HIV-1 Immunogen treatment was delayed in

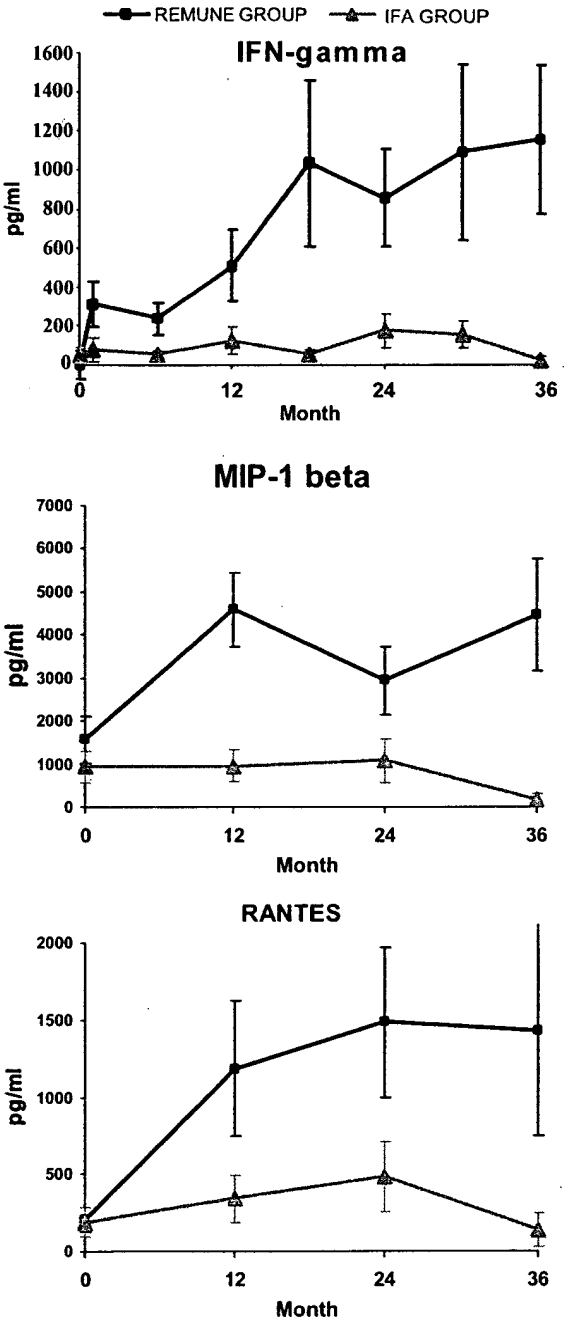

Fig. 4. Production of IFN-gamma and Beta-chemokines in PBMCs stimulated with HIV-1 antigens. Results are expressed in pg/ml.

comparison to patients assigned to placebo. There was a sustained difference between HIV-1 Immunogen and IFA arms in the overall unadjusted Cox model ($P = 0.056$). When baseline prognostic factors, such as viral load, were used as a covariate or when baseline viral load and CD4 cell counts were used as covariates, there were statistically significant differences between the groups with respect to the rate of virological failure ($P = 0.029$). Several studies of

HAART and ART, have shown that patients with high viral load prior to antiviral drug treatment had a more rapid failure rate than those with lower pre-ART viral loads [13,14]. Moreover, baseline CD4 T cell count has been observed to impact on the response to antiviral drugs alone and to other immune-based therapies [15–17]. In the assessment of a vaccine's effect on disease progression in an efficacy trial, it has been suggested that a vaccine demonstrating moderately durable effects to delay therapy and to ameliorate viremia merits consideration [18].

A previous study using the same HIV-1 Immunogen with different objectives did not find evidence of long-term clinical benefit of the HIV-1 Immunogen with different combination regimens of antiretrovirals [9]. The design of this trial differs mainly from our study, since in the assessment of the vaccine this study uses as the primary variable time to AIDS or death [18].

Induction of T cell help is critical in HIV-1 control and potentially in prevention by immunization [19]. A strong and persistant virus-specific T cell helper LPR has been observed in HIV-1 long-term survivors [20]. Other studies have explored the value of post-infection immunization in defining HIV-specific T helper effects and their in vivo HIV immunoregulatory relevance [21]. Improved short-term survival has also been demonstrated [22]. Our data and other reports have also shown that therapeutic immunization induces $CD4^+$ T-specific helper responses [23–26]. In addition to previous studies, we have shown equivalent responses between the two groups for mitogens and alloantigens which highlight the HIV antigen-specific immune response in the HIV-1 Immunogen Arm. In addition, the beta-chemokines RANTES and MIP-1beta, have been implicated as being some of the protective factors in the immune response against HIV infection [27]. Augmentation of HIV-1-specific LPR correlated with increased production of beta-chemokines and interferon-gamma only in the group of patients receiving therapeutic immunization.

Immunosuppressed individuals affected by chronic HIV-1 infection are characterised by an atypical immune system activation with gradual increase in HIV-1 RNA viremia and impairment of HIV-specific CD4+ T helper cell function that leads to a state of severe immunodeficiency with depletion of CD4+ and CD8+ T lymphocytes and HIV disease progression. Our study suggests the importance of applying a combined strategy of long-term immunization applied during long-term continued antiretroviral therapy to achieve a positive impact on the control of viral replication concomitantly with augmentation of HIV-specific memory T-helper cell responses.

Valuable insights into the protective mechanisms and approaches to therapeutic vaccine development have been gained with this immunotherapeutic HIV-1 Immunogen [28]. Further studies in larger populations of patients are warranted to better determine the impact of therapeutic vaccination on clinical outcomes and to further elucidate the role of specific immunologic and virologic responses in different patient populations.Significant cost saving, improved quality of life and reduction of morbidity and mortality can be anticipated, if long-term virological failure can be controlled by the patient's immune system [29,30].

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ABSTRACT IAS05

Results of the Spanish Phase II Trial with a Therapeutic Vaccine: Enhancement of CD4 and CD8 specific immune responses against HIV-1 antigens may allow control of viral load during antiviral drug treatment interruption in HIV-1$^+$ individuals treated with an HIV-1 Immunogen.

Objectives: In study STIR-2102, immunization with REMUNE showed improved maintenance of virologic suppression in patients on ART (Vaccine 2004; 22: 2966-73). As HIV-specific immune responses correlated negatively with viral load (VL), we hypothesized that this immunization may control viral rebound during an Analytical Treatment Interruption (ATI) study (REMIT).

Methods: REMIT is a, randomized, double-blind ATI study that included 39 patients who received REMUNE or IFA in STIR-2102 followed by REMUNE in an open label extension. Subjects eligible to interrupt ART (VL<2,000 copies/ml and CD4$^+$ >= 500 cells/µl for the last 12 months) were randomized to receive five injections of REMUNE (N=21) or IFA (N=18) over 48 weeks. An unmatched observational parallel comparative group (OPC) of 19 patients who discontinued ART voluntarily was included. This study had three primary endpoints: VL>55,000 copies/ml, ART resumption and CD4<350 cells/µl. CD4, VL, lymphoproliferative responses (LPR) and ELISpot responses to HIV-1 antigens were evaluated throughout the study.

Results: VL was lower at week 48 in REMUNE patients compared to IFA (mean 3.86 vs 4.64 Log 10; p<0.05). Patients receiving >24 injections of REMUNE showed longest mean time to composite endpoint (>24: 41.32 ± 5.16 weeks; <24: 32.88 ± 4.19 weeks; OPC: 32.00 ± 5.11 weeks). At week 48 in the REMUNE arm a low VL was associated with significantly higher levels of CD4$^+$ and CD8$^+$ central memory T-cells [r=(-)0.371, p=0.026 and r= (-) 0.422 p=0.01] and of LPR [r = (-) 0.618, p<0.001]. Significant increases in CD8$^+$ specific T cell responses against Gag were observed compared to the IFA and OPC group (REMUNE =2,593±545; IFA=1,324±238; OPC=172±50 spots/10^6 PBMC; p=0.006 and p=0.01 respectively).

Conclusions: Long term vaccination with HIV-1 immunogen may enhance host HIV-1 specific CD4 and CD8 T cell responses and result in a better control of plasma VL after ART withdrawal.

EXHIBIT E

Immune modulation in HAART-naïve, asymptomatic HIV-infected individuals undergoing therapeutic vaccination with HIV-1 whole killed vaccine

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Background. Use of agents that enhance HIV-specific immune responses with the goal of delaying initiation of antiretroviral therapy is being considered in HIV infection. Immunogenicity of a gp120-depleted, whole-killed HIV-1 vaccine consisting of HIV-1 antigen in Incomplete Freund's Adjuvant (IFA) (REMUNE®), was assessed in antiretroviral-naive HIV-1 infected subjects.

Methods. HAART-naïve asymptomatic subjects with HIV-1 RNA 10,000-40,000 copies/mL and CD4 counts 400-800 cells/µl received three injections of REMUNE (n=19), IFA (n=11), or saline (n=10) at weeks 0, 12, and 24. Immunovirologic parameters were evaluated at these three times and 4 weeks after the last injection (wk 28).

Results. Median absolute CD4 counts remained stable through week 28 in the REMUNE subjects, but declined both in saline and IFA treated subjects. Stabilization of CD4 counts in Remune patients was associated with increases in serum IL-7 and in naïve (CCR7+/RA+) CD4 T cells seen in REMUNE-treated individuals. In contrast, effector memory (EM: CCR7-/RA-) CD4 T cells were diminished at week in 28 in subjects that received REMUNE. IL-7 is required for thymopoiesis resulting in the generation of naïve T cells and is augmented during T cell reconstitution. The augmented serum concentration of IL-7 observed in Remune-treated patients suggests that thymic function is positively modulated by this immunomodulatory agent.

Conclusions. Immunotherapy with REMUNE is associated with changes in circulating lymphocytes phenotype and, possibly, with a positive effect on thymic functions. Data from larger cohorts of subjects are required to assess clinical significance of these observations.